STUDIES ON THE PHYSIOLOGY OF *ARENICOLA MARINA* L.

II. ACCOMMODATION TO MAGNESIUM CONCENTRATION IN THE ISOLATED EXTROVERT

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(*Received* 10 *February* 1940)

(With Seven Text-figures)

OF the large number of published studies on the ion relations of invertebrate rhythmic muscles, the great majority concern preparations from Crustacea and Molluscs—largely because the animals of these groups are provided with vigorous and experimentally convenient hearts. Data on members of other phyla are relatively few and, from the comparative point of view, their multiplication is evidently desirable. Recently, one of us published a description of the so-called "isolated extrovert" of the lugworm, *Arenicola marina* L., a preparation consisting of the proboscis and part of the oesophagus (Wells, 1937). Suspended in sea water, the isolated extrovert maintains for many hours a very regular and characteristic activity pattern, consisting of a series of outbursts of vigorous rhythmical contraction separated by periods of relative rest. That this is the normal activity pattern was shown in the paper already mentioned. Because of its vigour, its regularity, its long survival after excision and the ease with which that operation is performed, it is an excellent object for experiments on the action of chemical factors. The writers have undertaken a study of ion actions on this preparation. The results obtained by varying the Mg concentration form the subject of the present communication. Experiments with K and Ca have been begun and it is hoped to complete them shortly.

METHODS

The dissection of the isolated extrovert was carried out as already described (Wells, 1937, p. 122). Care was always taken to leave the circum-oesophageal nerve ring and supra-oesophageal ganglia out of the preparation, as their presence may introduce irregularities into its behaviour. It is of great importance, when dissecting out an extrovert, to avoid stretching the tissue, especially in the region where oesophagus joins the proboscis, near the insertion of the retractor muscle into the gut wall. The movements were recorded with very light isotonic levers.

The preparations were exposed to Mg concentrations which changed either abruptly ("*constant exposure*" experiments) or gradually ("*drift*" experiments) from one value to another, using methods described elsewhere (Wells & Ledingham, 1940).

All solutions applied to the extroverts were made of A.R. reagents, with watt from an ordinary block tin condensing tube. We have not attempted to work wi any preparations for much longer than 24 hr., and in experiments of this duratio further precautions against traces of impurities do not seem to be necessar Solutions were made up in distilled water to which Na bicarbonate $N/400$ had bee added, and which had been vigorously aerated overnight. The resulting salines we adequately buffered at *p*H 8·0–8·2, i.e. near the normal *p*H of sea water.

It is already known that sea water keeps the extrovert in a vigorous and a parently normal condition for a long time (Wells, 1937), and we started with th assumption that a balanced solution closely resembling sea water in compositio would be a normal medium. Reliable analyses of *Arenicola* blood or body fluid are n available. The artificial sea water was made by mixing a number of solutions ea approximately isotonic with sea water (McClendon, 1916; Pantin, 1926), so that th proportions of the various ions could readily be varied when desired, without great altering the osmotic pressure of the resulting mixture. The proportions finall adopted were as follows:

Potassium chloride	0·6 *M*, 1·8 c.c.
Calcium chloride	0·4 *M*, 2·8 c.c.
Sodium sulphate	0·55 *M*, 5·5 c.c.
Magnesium chloride	0·4 *M*, 14·5 c.c.
Sodium chloride	0·6 *M* (to 100 c.c.)

This mixture supported the rhythm as well as natural sea water, and chang from one to the other produced no obvious modification in activity. In making u solutions with "abnormal" Mg concentrations (it should be remembered that th "normality" of the above saline is assumed, simply because the mixture works) th proportion of $MgCl_2$ taken was varied as desired. Since all mixtures were made u to 100 c.c. with NaCl, variation in Mg entailed an opposite variation in Na. I other words, the following factors were held constant throughout the work: K, C sulphate, *p*H, osmotic pressure. To ensure constancy of the latter factor, increase i Mg was compensated by decrease in Na, and vice versa. The inclusion of sulpha as the sodium, instead of the more usual magnesium, salt obviously facilitate matters when low Mg concentrations are wanted.

To simplify the statement of Mg concentrations, the "normal" saline, whos composition is given in detail above, and which was used as the starting point in ou experiments, is referred to in the following pages as "Mg 1", and other solution are characterized simply by their Mg content, which is given in multiples of that i the "normal" mixture. Thus "Mg 2", for example, is a mixture containing twic as much Mg as artificial sea water, and so on. "Mg 1" contains 0·058 *M* MgCl.

RESULTS

The essential facts can be summarized in two statements: (1) Mg tends t inhibit the preparation; (2) the preparation has a considerable power of accommoda ing itself to a new Mg concentration, so the effects of a sudden change in the amou

of Mg in the bathing fluid are greatest at first, then gradually become less. These points are brought out in Figs. 1, 2.

Fig. 1 shows that on changing from Mg 1 to Mg 2, the extrovert is at first completely relaxed. In four hours' exposure to the high concentration, a considerable degree of recovery has occurred (accommodation to high Mg). On returning to

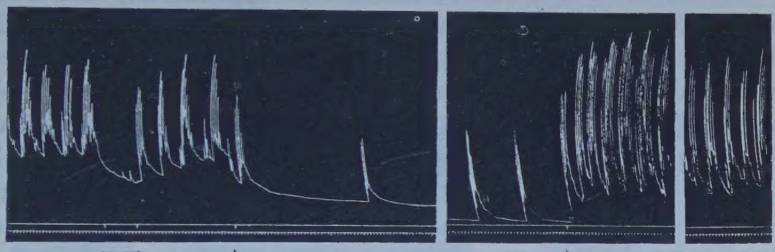


Fig. 1. Accommodation to a high Mg concentration. First extract begins in Mg 1, and shows a short exposure (10 min.) to Mg 2, and the beginning of a long exposure (4 hr.), also to Mg 2. Second extract shows return to Mg 1 after the long exposure. Third extract shows behaviour of the preparation, still in Mg 1, 2 hr. later. *In all records:* read from left to right; upstroke of lever means contraction of preparation; time signal marks minutes; Mg concentrations given as multiples of that in sea water.

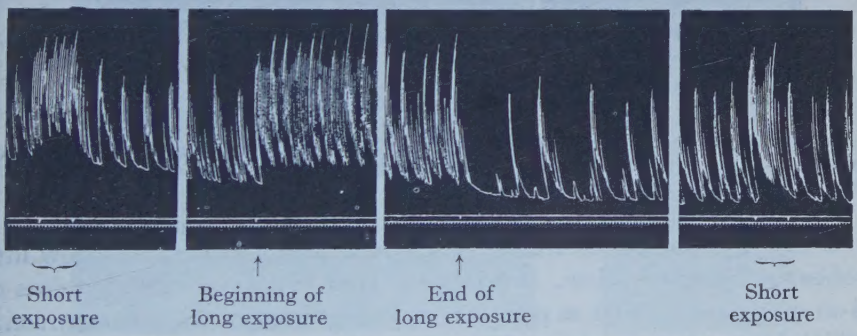


Fig. 2. Accommodation to a low Mg concentration. First extract begins in Mg 1, and shows a short exposure (10 min.) to Mg 0·5. Second extract shows the beginning of a long exposure (4 hr.), also to Mg 0·5. Third extract shows return to Mg 1 after the long exposure. Fourth extract shows behaviour of preparation, still in Mg 1, 3 hr. later; also a second short exposure to Mg 5.

Mg 1, the effect depends on the duration of the exposure to Mg 2. After a short exposure, during which the extrovert had no time to adjust itself to the new conditions, the original behaviour pattern is resumed smoothly and directly on returning to Mg 1. But after long exposure, when accommodation has occurred, return to Mg 1 causes hyperactivity, i.e. the typical reaction to low Mg. This gradually passes off (accommodation to Mg 1).

Fig. 2 shows the corresponding experiment with a low Mg concentration. On changing to Mg 0·5, there is an increase in activity, which passes off to a large extent if the extrovert is given time to accommodate itself to the new conditions. On

returning to Mg 1, the result is a temporary inhibition—i.e. the typical reaction to high Mg—if, and only if, the exposure to Mg 0·5 was long enough for accommodation to occur.

Accommodation, then, can be detected in two ways. As an extrovert adjusts itself to a new solution its behaviour improves, but at the same time the old solution ceases to be appropriate. The immediate effect of applying any mixture depends on the previous history of the preparation; thus Mg. 1 causes excitement in Fig. 1 and inhibition in Fig. 2.

These accommodation phenomena are sometimes exceedingly striking. With small changes—e.g. Mg 1 to Mg 1·5 or to Mg 0·75—the difference between the immediate response and the final equilibrium condition is not great, but with such changes as Mg 1 to Mg 3, or Mg 1 to Mg 0, the activity may be completely suspended, or very greatly increased, for many hours before accommodation results in comparatively normal behaviour.

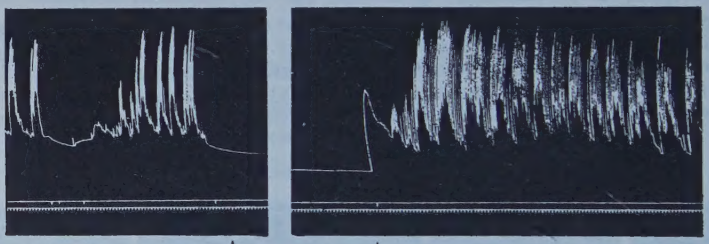


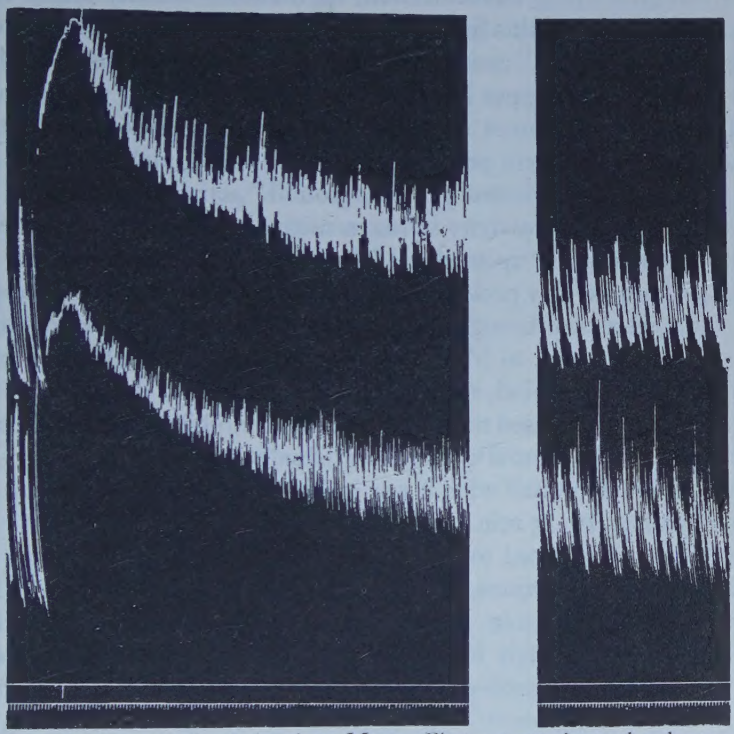
Fig. 3. Accommodation to a concentration of Mg, so high that activity is impossible. First extract begins in Mg 1, and shows a short exposure (10 min.) and the beginning of a long one (4 hr.) to Mg 6·2. Second extract shows return to Mg 1 after the long exposure. Compare Fig. 1.

About the physiological nature of the accommodation process we know little. The problem is discussed below. It is however clear that accommodation does not depend on special events, such as permeability changes, associated with contraction. In very high Mg concentrations, spontaneous activity never appears, but the accommodation process can nevertheless be shown to take place. Fig. 3 illustrates an experiment in which the extrovert was alternated between Mg 1 and a solution containing no NaCl, the necessary amounts of KCl, $CaCl_2$ and Na_2SO_4 being made up to 100 with $MgCl_2$. This gave a Mg value of 6·2. It will be seen that return to normal causes great temporary excitement after long exposure to high Mg, but not after short. This proves that the accommodation process has taken place, although in the presence of so much Mg it could not lead to resumption of mechanical activity. Incidentally, it is interesting to note that Mg inhibition is promptly and completely reversible, even with such high concentrations. After recording the second half of Fig. 3, we put the preparations—a group of four, of which the record of one is shown—back into Mg 6·2 and left them in it, at room temperature, for 15 hr. After this time they were completely limp and toneless, and we thought them dead. They were

owever mounted for recording, and, on returning them to Mg 1, there was im-
ediate recovery of tone followed by great hyperactivity slowly settling down into
ne typical pattern, very much as in the second half of Fig. 3.

The effects of different Mg concentrations will now be described in fuller detail.

Over the range from Mg 0 to Mg 1, the chief effects of variation in Mg concen-
ation are as follows. Normally, the extrovert exhibits a regular series of activity
utbursts, each consisting of a tone wave upon which a number of vigorous rhythmic
rokes are superposed. If the Mg is high, the period of rhythmic strokes tends to be



ig. 4. Two extroverts exposed, at signal, to Mg 0. First extract shows the change and the first 2 hr. of the exposure. Second extract shows behaviour 6 hr. later, still in Mg 0.

onfined to the top of the wave. If the Mg is low, the active period spreads over the
hole wave and even into the intervals between the waves. The general tone level is
epressed by high, and raised by low, Mg. A big downward change, e.g. from Mg 1
o Mg 0, produces violent, tetanus-like contraction, with minute strokes superposed
n it, but even in the total absence of Mg, the contraction slowly passes off and, in a
ew hours, the typical intermittent behaviour pattern can be seen again (Fig. 4).

More intense Mg inhibition can be seen in extroverts equilibrated with high Mg
oncentrations (e.g. Mg 2 upwards), or, at lower concentrations, as temporary
ffects following such sharp upward changes as Mg 0 to Mg 1. In these conditions,
ne interval between successive outbursts lengthens, and the amplitude of the con-
actions may diminish.

It seems probable, from the general nature of these effects, that the action of M is primarily on the excitor mechanism. We do not seem however to be dealing with a simple action on one site, for the type of effect produced by raising the Mg varie with the Mg concentration. Confining our attention to extroverts in equilibriu with their bathing media, we find that, over the lower part of the concentratio range, increasing Mg restricts the extent of each activity period; that at higher M concentrations, increase lengthens the interval between outbursts; and that at sti higher concentrations, the amplitude of the strokes falls off.

Above a certain limiting concentration, spontaneous activity ceases altogethe The exact determination of this limit is however difficult. Our first experiments o this problem (made with the "constant exposure" technique) established the follow ing points: (1) there is an upper limit, apparently at about Mg 3, above which th normal outburst pattern cannot appear; (2) there are considerable differences i Mg tolerance between different preparations; (3) under normal conditions, prepara tions often show a certain amount of "background" activity between the rhythm outbursts, this background activity is less sensitive to high Mg than the outbur pattern and may be present up to about Mg 5; (4) temporary inhibition followin upward changes may be very prolonged, especially as the upper limit is approache

These points were well brought out in an experiment on ten extroverts, whic were exposed, first for 4 hr. to Mg 1 and then for 19 hr. to higher Mg concentra tions. During the latter period, they behaved as follows.

Three preparations exposed to Mg 2·5 were first inhibited for periods of 2–4 hr and then comparatively normal outbursts gradually returned. However, the fin frequency remained about half what it was in Mg 1, the intervals between outburs now occupying about 10–15 min.

Four preparations exposed to Mg 3 were inhibited for 7–8 hr., then outburst reappeared. In two preparations, the outbursts, although weaker than in Mg 1 an occurring at intervals of 20 min. or more, were still fairly vigorous. In the other the outbursts were exceedingly feeble and occurred at very long intervals.

Three preparations exposed to Mg 3·5 showed complete inhibition for sever hours, after which "background" activity gradually returned. No distinct outburst appeared during the whole of the 19 hr. exposure. One preparation showed a singl brief tone wave near the end of this period, which was perhaps an abnormal out burst.

The latter observation raises the question, if the exposure to Mg 3·5 had bee longer, would the extroverts have become active again? To attempt to answer th question by further prolonging the experiment would be hardly justifiable. A described above, it already involves continuous recording of the excised preparation for 23 hr. It is not possible to use extroverts for much longer than this, owing to th intervention of weakness, irregularity and other results of the long survival perio If the question is to be answered, it must be in some other way.

We therefore carried out a number of "drift" experiments, in which the M concentration changed very gradually, using methods described in detail elsewhe (Wells & Ledingham, 1940). In some cases, the Mg started at the sea water con

entration and was made to rise slowly. In others, the extroverts were first accom-
nodated to Mg concentrations well above the limit and then exposed to conditions
n which the Mg gradually fell towards the sea water value. The object of these
xperiments was to avoid temporary effects of quick change. If the rate of drift is
low enough, the tissue will be able to keep itself accommodated all the time. Failure

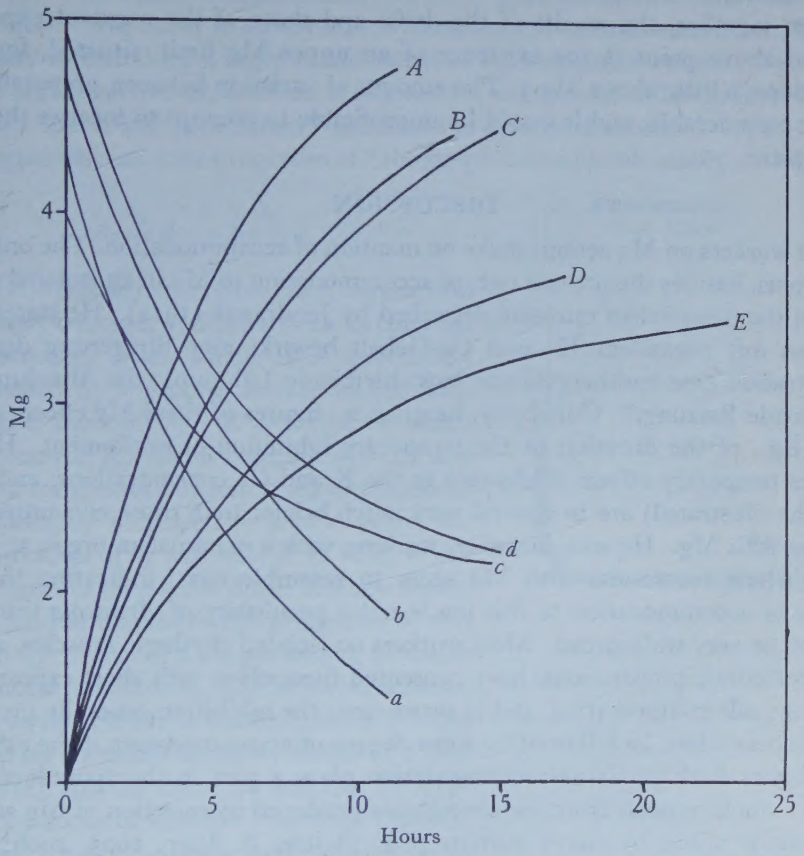


Fig. 5. Graphs of the drift experiments, to show how the Mg concentration varied with time.

o drift slowly enough will be revealed by a difference between the limits shown by
pward and by downward drifts; for an upward drift, by inhibiting the cells, will
ield a low limit, while a downward drift will have the opposite effect.

Fig. 5 gives the Mg time curves of our various drifts. Each curve represents a
ingle experiment made on two or (more usually) three extroverts simultaneously.
Upward drifts A, B, C were too rapid; the extroverts were inhibited, more or less
harply, at concentrations varying from Mg 1·5 to 2·5. Upward drifts D, E were
atisfactory; at Mg 3, the preparations were still giving quite vigorous outbursts,
lthough at long intervals, but above this value the falling away was marked. At
he ends of D and E outbursts still occurred at very long intervals, but they had

degenerated into brief tone waves without superposed strokes, and were usually so small as to be nearly lost in the "background" activity of the preparation. The downward drifts (*a–d*) were apparently a little too fast; the extroverts became active, rather abruptly, in the range Mg 2·9–3·6. In this case, as already stated, a slight raising of the apparent limit is to be expected, since a downward change has an exciting effect.

Taken together, the results of the drifts and those of the constant exposures described above point to the existence of an upper Mg limit, situated, for most preparations, a little above Mg 3. The amount of variation between preparations is however considerable, and it would be unprofitable to attempt to localize the limit more exactly.

DISCUSSION

Most workers on Mg actions make no mention of accommodation. The only case known to us, besides the present one, of accommodation to Mg in an isolated organ is that of the mammalian intestine described by Jendrassik (1924). He states: "In Lösungen mit normalem K- und Ca-Gehalt bewirkt eine Steigerung der Mg-Konzentration eine vorübergehende bzw. bleibende Lähmung, die Abnahme von übergehende Reizung." Unhappily, he gives no figures of these Mg effects and no details, e.g., of the duration of the temporary inhibition or excitement. He also describes temporary effects of changes in the K and Ca concentrations, and these (which he illustrated) are in general very much briefer than those encountered by ourselves with Mg. He was, however, working with a mammalian organ at 37° C. On the whole his results with Mg seem to resemble ours, indicating that the property of accommodation to this ion is not a peculiarity of *Arenicola* tissue. It may even be very widespread. Most workers on isolated rhythmic muscles, at least with invertebrate preparations, have contented themselves with short exposures to the various salt mixtures tried, and in many cases the inhibition generally produced by Mg excess might be followed by some degree of accommodation if the exposure were longer. Perhaps tissue-accommodation plays a part in the spontaneous recovery of whole animals from the anaesthesia produced by injection of Mg salts—a phenomenon noted by many authors (e.g. Meltzer & Auer, 1905, 1906; Wiki 1934).

Turning now to the problem of the mechanism of accommodation, the fact that a sudden change produces (1) immediate response, followed by (2) slow adjustment suggests at once that the first is a direct action of the new chemical environment on the surface of the cell, while the second is due to the penetration of some substance across the cell membrane. This is the viewpoint adopted by Jendrassik (1924). The substance which actually crosses the membrane need not necessarily be Mg. One can assume, on the one hand, that excitability depends in some way on the relative amounts of Mg within and without the cell, in which case Mg penetration will cause accommodation, or, on the other hand, that increased external Mg lowers excitability by entering into reversible combination with some organic constituent of the cell surface, whose uncombined fraction is then partly restored by slow outward diffusion from reserves within the cell. Both hypotheses have an essential point in

common—they ascribe the slowness of accommodation to the difficulty of penetrating the cell membrane.

While there is at present no critical experimental evidence for or against such hypotheses, it should be emphasized that the experimental results can be otherwise explained. In a recent theoretical discussion of the properties of the steady state compared with those of equilibrium, Burton (1939) has shown that what he calls "overshoot" will occur in steady state systems under certain conditions. It is only necessary to introduce a slight modification of his theoretical system, to arrive at a simple and attractive explanation of our results, along quite different lines from the "surface action and penetration" mechanisms described above.

Burton discusses the properties of "the simplest imaginable steady state system", i.e.

$$S \overset{k_0}{\longleftrightarrow} A \overset{k}{\longrightarrow} B \overset{k_z}{\longleftrightarrow} Z$$

where $A \rightarrow B$ is an irreversible chemical reaction of velocity constant k; S is a source, of constant concentration, from which A is continually replenished by a process similar to diffusion (diffusion constant k_0); Z is a sink to which B is removed (diffusion constant k_z). If, as a result of some external agency, velocity constant k is suddenly increased to a new value, the concentration of B will rise; if $k_0 < k_z$, it will overshoot and then settle down to a new steady level. Burton illustrates the properties of this system by means of a simple model, in which the concentrations of S, A, B, Z are represented by the levels of water in vessels (that of B being written on a smoked drum by means of a float and lever), and the diffusion and reaction constants by cocks of variable resistance. He also discusses more complicated steady state systems, and the conditions under which they will overshoot.

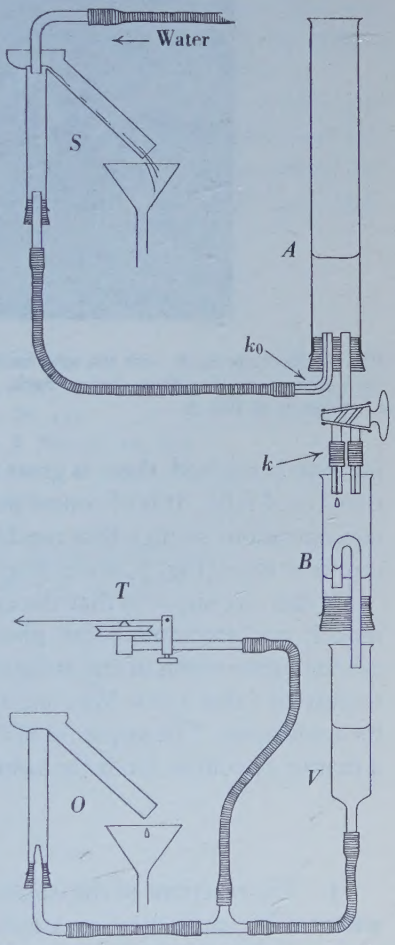


Fig. 6. Water model to show accommodation in a rhythmic steady state system.

Suppose now that, instead of diffusing away to Z, B behaves as a relaxation oscillator, and accumulates until a critical concentration is reached, when it is suddenly and completely removed. The result will be a rhythmic system in which the frequency of the rhythm behaves as the concentration of B does in Burton's case. The frequency will rise and fall with k, and overshoot after a sudden change in the value of that constant.

To make this clear, we have constructed a water model, similar to Burton's as regards S and A, but with a relaxation oscillator for B (Fig. 6). Velocity constant k

is represented by either of two capillaries of different diameters, so its value can b changed by turning the two-way tap. To emphasize the analogy with a rhythm muscle, *B* may be called the "excitor mechanism", and vessel *V*, overflow *O* an tambour *T* may together be called the "contractile mechanism", since their fun tion is to inscribe a contraction of appropriate form on the drum for every discharg of *B*. The apparatus gives records closely paralleling those of the extrovert (Fig. 7 If the system is running steadily with a fast capillary at *k*, the level of *A* will b fairly low. If a slow capillary is now substituted (Fig. 7, upper line), the flow ra into *B* falls at once, but it then shows a gradual "accommodation" as the level of rises to a new steady position. On returning to the original capillary after the ne

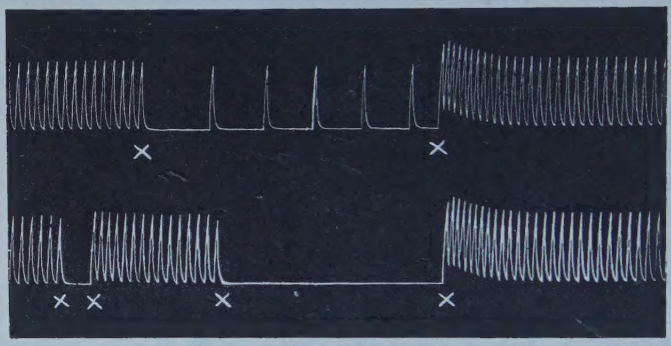


Fig. 7. Records made with the apparatus of Fig. 6. Tap *k* changed at *X*. Upper line: fast flow rat then change to slow, then change back. Lower line: imitation (by temporarily closing tap *k*) of th experiment of Fig. 3.

position is reached, there is great acceleration followed once more by "accommod tion" as *A* falls. It is of course possible to imitate the action on the extrovert of M concentrations so high that mechanical activity is impossible, by simply closing th tap for a time (Fig. 7, lower line).

If then we suppose that the extrovert is a steady state system comparable to th model, our accommodation phenomena can be explained without assuming th gradual penetration of any substance across the cell membrane. It is only necessar to postulate that a new Mg concentration alters the value of *k* as from the moment o its application. The sequence of "overshoot" and "accommodation" will follow a matter of course, from the resultant changes in concentration of substance *A*.

SUMMARY

1. The reactions of the isolated extrovert of *Arenicola marina* to variations in th external Mg concentration are described and discussed.

2. Artificial sea water supports a vigorous rhythm for many hours and wa therefore arbitrarily taken as the "normal" saline. In all mixtures, the followin were held constant: *p*H, K, Ca, sulphate, osmotic pressure. Increase in Mg wa osmotically compensated by decrease in Na, and vice versa.

3. High Mg concentrations depress, and low ones raise, the spontaneous activity vel of the preparation.

4. The preparation can accommodate itself, to a large extent, to a new Mg ncentration. The effects of abruptly changing to a new mixture are greatest just ter the change, and gradually become less as accommodation occurs.

5. After accommodation to a new Mg concentration, the old is no longer ppropriate. Return to artificial sea water evokes Mg-deficiency reactions after ccommodation to high Mg, and Mg-excess reactions after accommodation to low Ig. In either case, the preparation slowly accommodates itself back again to normal.

6. The accommodation process occurs in mixtures whose Mg concentration is high that spontaneous activity cannot reappear. This is shown by the fact that Ig-deficiency reactions are evoked by changing back to normal after long exposure such mixtures, and proves that accommodation does not depend on special vents (such as permeability changes) associated with functional activity.

7. If time enough for accommodation is allowed, or if the change of Mg con-ntration is made very slowly ("drift" experiments), it is found that fairly normal ctivity can occur over a wide range—from Mg-free mixtures to mixtures containing bout three times the amount of Mg in sea water. At the upper end of this range, ie preparations are, however, markedly depressed.

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THE DIGESTIVE ENZYMES OF SOME WOOD-BORING BEETLE LARVAE

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(*Received* 10 *May* 1940)

(With One Text-figure)

INTRODUCTORY

The digestive powers of insects living in wood have long been a subject of intere but it is only during the last ten to twelve years that a real attempt has been ma to investigate the physiology of digestion of these organisms. The lack of resear upon this problem is probably to be explained by the difficulty of obtaining a quate supplies of insects and of experimenting with organisms feeding upon substance of such complex chemical constitution. A wide field for investigati still remains, but sufficient work has been published to enable the trend of r results to be appreciated. It is of the utmost importance that research should undertaken upon as many species of wood-borers as possible, since it is alrea evident that there exists considerable diversity in the ways in which insects der their sustenance from wood.

Uvarov (1928) in his summary of the literature up to 1927 on insect nutriti concluded that the food relationships of the vast majority of wood-eating inse were absolutely unknown, but he overlooked two important papers by Mer (18 1903), who had produced evidence to show that certain beetle larvae (*Lyctus* sp are dependent upon the starch in wood. Another review of knowledge of the dig tion of wood by insects was made in 1934 by Mansour & Mansour-Bek (1934 who were able to consider, in addition to their own experiments (1934*a*), the resu of several investigators who had worked in the interim. They distinguished betwe two types of wood-eating insects, those secreting a cellulase and those unable do so. The former derive nourishment mainly from the cell-wall componen whilst the latter are dependent upon the cell reserve materials. More rec information (Hopf, 1938) suggests that wood-feeding beetle larvae may be divisi into three classes on the basis of the complexity of the wood components wh they hydrolyse during digestion.

In spite of the work so far carried out, however, only the fringe of the probl of wood-feeding has been investigated. For instance, the restriction of ma species of wood-borers to a limited range of hosts has not yet been explained a information upon the relation between larval nutritional requirements and h specificity must be of interest. In this connexion the demands of the larva fo supply of suitable food material may be reflected in the tropisms of the bee

hen selecting a site for oviposition. For example, Parkin (1936) has demonstrated at the need of *Lyctus* larvae for starch is met by the ability of ovipositing females recognize the presence of this substance in wood and that, in consequence, eggs e not laid in starch-free timber. Furthermore, the role of symbiotic micro-ganisms in digestion is not yet settled beyond question. Unlike the Protozoa the digestive tracts of most Termites, the symbiotic yeasts in wood-boring beetle rvae appear to play no direct part in the digestion of wood, but Koch (1934) has ade the very interesting discovery that larvae of *Sitodrepa panicea* L., an Anobiid ing on stored foodstuffs, are greatly stunted in growth when artificially rendered ee from symbionts, and that normal growth can be restored by addition to the od of yeast or wheat germ. If the active principle of the yeast and wheat should ove to be a vitamin, the knowledge may prove valuable in relation to experiments on the vitamin requirements of wood-borers, a subject which has not yet been vestigated.

Up to the present, research upon the food relations of wood-boring coleopterous rvae has followd four main lines, namely:

(i) chemical analysis of food and excrement;
(ii) removal of certain substances from the wood by extraction with solvents;
(iii) feeding on artificial diets;
(iv) testing for the presence of enzymes in the gut.

These experimental methods are clearly complementary and the proper correlation of the results obtained should yield the answer to the question, how can sects satisfy their nutritional requirements from a diet of wood. The value to plied entomology of such fundamental knowledge is illustrated by the work of e author and others upon the food relations of *Lyctus* powder-post beetles, od-borers of considerable economic importance. It has been shown that starch wood forms the principal food material for the larvae and that female beetles oid oviposition in starch-free timber. This knowledge has led to research upon ethods of rendering wood free from starch as a means of preventing infestation *Lyctus*.

The investigation dealt with in this paper has been concerned with tests for e presence of certain enzymes in the guts of a number of species of wood-boring etle larvae, and an attempt has been made to relate the results to our present owledge of the chemistry of wood and to the work of other investigators. The periments have been confined to Coleoptera able to feed in bark or wood which is und or but slightly decayed, and the term "wood-borer", for the purpose of this per, does not include such forms as can live only in wet wood in an advanced ge of fungal decay. There is, of course, no hard and fast line of demarcation tween the two groups.

THE COMPONENTS OF WOOD

Before dealing with the experimental side of the work, it seems advisable to give brief account of the composition of the substrate upon which wood-boring insects d. Chemical analysis of wood is usually undertaken according to a series of

standard methods which enable the amounts of the principal components to measured. The meaning of the resulting data is often not clearly understood biologists owing to the difficulty of realizing the relation between substances isolat or estimated by chemical methods and their counterparts incorporated in structure of the wood. For our purpose, we may consider wood to be formed minor and major components according to whether they are present in small large amounts.

The minor components include the cell contents, which are generally remova from finely divided wood by neutral solvents and consist of such materials tannins, resins, dyes, alkaloids, fats, oils, gums, sugars, starch, proteins and mine salts. By prolonged extraction with boiling water, Campbell (1935) has isolat from oak sapwood substances which he terms "amylo-uronides". These are hyd lysed by takadiastase to yield glucose, but they also contain some xylose a uronic acid units and are probably intermediate in composition between starch a certain hemicelluloses.

The major components, which usually comprise about 90 % of the wo substance, are cellulose, lignin and hemicelluloses. The modern biochemical co ception of the fine structure of the cell wall is explained in a review by Clarke (193 Cellulose molecules are aggregated into micelles which may be regarded as the ba units forming the framework of the cell wall. On this framework are encrust lignin and hemicellulose in such a way as to form a second continuous and int penetrating system. These two systems cannot at present be separated by chemi analysis without causing some constitutional change in the desired product.

In the present state of knowledge, the cellulose molecule is to be regarded a chain of anhydro-glucose units united through β-linkages and starch as the cor sponding substance with α-linkages. Owing to its more symmetrical spatial co figuration, the cellulose molecule is more stable than that of starch. In spite of large volume of research which has been carried out to ascertain the constitut of lignin, little more is known than that it is probably largely aromatic in charact Similarly little is known about the composition of the hemicelluloses. Some app to be closely associated with the cellulose and in most wood analyses are estima as "pentosans in the cellulose". O'Dwyer (1937, 1939, 1940) has recently b able to show that hemicelluloses A and B of oak heartwood have a common recurr structural unit consisting of anhydroxylose and methylhexuronic anhydride un in the proportion of 6:1 respectively. The corresponding products from sapwo have as an additional constituent a variable number of anhydroglucose units. Th must presumably be some difference in degree of aggregation of the molecules the two hemicelluloses to account for the greater solubility of B, which sho render it more easily available to wood-feeding insects.

Chemical analysis unfortunately does not enable the absolute quantities of various components as they occur in the wood to be estimated. For instance, isolation of cellulose by delignification of wood with chlorine and sodium sulph yields "Cross and Bevan cellulose", a product containing α-, β- and γ-cellulos There is no evidence to show that β- and γ-celluloses occur in such as wood

ct β- and γ-celluloses are probably derived from α-cellulose during the chemical eatment. Similarly, hydrolysis of all the carbohydrates of wood with 72 % lphuric acid leaves a residue called lignin by wood chemists, but which is gener-ly held to be different from the lignin occurring naturally in the cell wall. How-er, by the use of standardized methods, chemical analysis gives results which are pable of repetition and can therefore be used to demonstrate changes in the com-osition of wood and to indicate which components have been most affected.

One of the main methods of approach to the problem of wood feeding has been e comparison of chemical analyses of the wood in which an insect is tunnelling d the bore-dust remaining in the galleries. This method reveals whether there s been any change in the proportion of the various wood components during the rocess of digestion, but cannot show the amount of each component eaten unless e weight of the wood sample is known before and after attack. Most workers have ied to overcome this difficulty by assuming that the ash or lignin content of the ood is not affected during passage through the larval gut. From spectrographic alysis and X-ray photography of *Lyctus* larvae in wood, however, Jones & itchie (1937) have shown that mineral constituents are absorbed by the insects. urthermore, it is well known that wood-destroying fungi can break down lignin, d until proof is available that insects cannot also do so, the assumption that the gnin is not altered during larval digestion is hardly justifiable. The chemical alyses so far published are not rendered valueless by being based on this assump-on, however, since the figures obtained represent the minimum changes which ve occurred. Proof of the decomposition of lignin during digestion would there-re mean that a higher proportion of the other constituents is utilized than has therto been revealed.

MATERIAL

The larvae used in this investigation were obtained principally from the stocks infested wood in the insectaries at the Forest Products Research Laboratory or om samples submitted for examination as part of the advisory work of the aboratory. Identification of the insects was based upon knowledge of the type of jury caused in timber, examination of the morphological characters of the larvae d comparison with such descriptions and keys as are available, and, where ssible, identification of beetles associated with or reared from the larvae. Data the species used and the origin of the timber in which they were found are mmarized below.

Scolytidae

olytus destructor Ol.	Larvae and beetles from elm bark. Princes Risborough, 1937.
hloeosinus bicolor Brull.	Larvae and beetles from African pencil cedar bark. Imported from Kenya, 1936.

* I wish to record my thanks to Dr K. G. Blair, British Museum (Natural History), for the ntification of these beetles.

CERAMBYCIDAE

Rhagium mordax De G.	Larvae under oak bark. Forest of Dean, 1939.
Phymatodes testaceus L.	Larvae bred in oak bark. Princes Risborough, 1936.
**Isotomus speciosus* Schneider	Larvae and beetles from hornbeam. Imported from Fran 1936.
Xylotrechus rusticus L.	Larvae and beetles from aspen. Imported from Finland, 193
†*Hylotrupes bajulus* L.	Larvae from Scots pine. Sent from Copenhagen, 1938.
Smodicum cucujiforme Say	Larvae and beetles from red oak. Imported from U.S.A., 193

LYCTIDAE

Lyctus brunneus Steph.	Larvae bred in oak. Princes Risborough, 1933–6.

BOSTRYCHIDAE

Heterobostrychus brunneus Murr.	Larvae and beetles from obechi (mixed infection). Import
Bostrychoplites cornutus Ol.	from Nigeria, 1938.

ANOBIIDAE

Anobium punctatum De G.	Larvae and beetles from beech. Princes Risborough, 1933.
Xestobium rufovillosum De G.	Larvae bred in willow. Princes Risborough, 1933–6.
Ernobius mollis L.	Larvae and beetles from spruce bark. Princes Risborou 1937.
Ptilinus pectinicornis L.	Larvae and beetles from beech and sycamore. Princes R borough, 1933–7.

METHODS

Throughout the investigation only qualitative tests for digestive enzymes we undertaken and the technique finally adopted was based on work by Swingle (192 and Wigglesworth (1927). Special attention was paid to the occurrence of carb hydrases; a test for proteinase was also carried out, but no satisfactory test f ligninase applicable to small quantities of insect digestive juices could be d covered.

Various methods have been used by different workers in order to detect t presence of a cellulase in the alimentary canal of wood-boring larvae, and some the results must be accepted with caution, pending confirmation. The reliabili of the test involving breakdown of filter-paper or cotton-wool is dependent up the purity of the material with regard to α-cellulose content. Moreover, unless t difference is considerable, it is not safe to assume the presence of a cellulase fro cellulose determinations of food and frass by the Cross and Bevan method analysis, owing to the presence in the cellulose of pentosans which may occur impurities in quantities up to 20 %, especially in hardwoods. The occurrence so high a proportion of pentosans in Cross and Bevan cellulose also precludes t use of this substance as a substrate for tests with enzyme solutions. The mo reliable tests would appear to be the breakdown of α-cellulose *in vitro* or the d appearance of cellulose from sections of plant tissue as shown by the chlor-zi

* I wish to record my thanks to Dr K. G. Blair, British Museum (Natural History), for identification of these beetles.

† I am indebted to Konsulent H. Wichmand, Teknologisk Institut, Copenhagen, for th specimens.

dide reaction; α-cellulose prepared from oak was therefore used as the substrate the present work.

Larvae were cut from the wood not more than one day before they were equired, 10–150 being used for each experiment according to size. The guts were issected out and freed, so far as possible, from fat body, tracheae, Malpighian ubes, etc. They were then transferred to an agate mortar containing about 3 c.c. of reviously boiled distilled water to which a drop of toluene had been added. The uts were broken up by teasing with needles and were then ground with a small uantity of clean sand. The sand was allowed to settle and the supernatant fluid ipetted off: more water was added and the process repeated until about 10 c.c. of ilute digestive fluid and tissue suspension were obtained. The liquid was divided to two equal portions, one of which, after heating in a boiling water-bath for 15 min. inactivate the enzymes present, was used as control. As soon as they were pre-ared, both suspensions were covered with a film of toluene.

Glass tubing, 3 mm. in internal diameter, was drawn out as shown in Fig. 1 form at one end an ampoule about 2·5 cm. long. By means of a rubber teat, the mpoule was one-third filled with enzyme suspension, when substrate (1 %

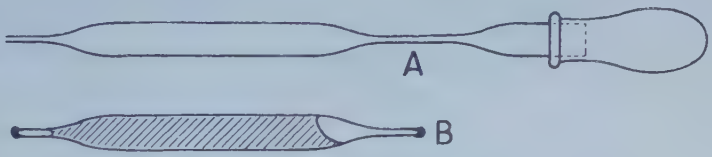

Fig. 1. A, ampoule ready for filling; B, filled and sealed.

luble starch, 3 % sucrose, 3 % maltose, or 3 % lactose) was sucked in until only small air bubble remained. After breaking off the extension of the ampoule bearing e rubber teat, the ends were sealed in a flame, and by repeated inversion and aking the bubble was made to mix the contents thoroughly. With solid substrates -cellulose, hemicelluloses A and B*) a small quantity was inserted into the mpoule after drawing out the first end. A similar set of ampoules was made with e boiled fluid to act as controls. Both sets were then maintained at 37° C.

The preparation of 10 c.c. of suspension allowed each test to be made in uplicate, one of the ampoules being opened after approximately 70 hr., and the her after 140 hr. After breaking off the ends of the ampoule, the contents were pelled into a small test tube and tested for the presence of reducing sugars by iling with Fehling's or Barfoed's reagents. The intensity of copper reduction, ence the activity of the ferment, was recorded approximately by one to four osses (see Table I). It must be remembered that comparison of the activity of e enzymes in the different species of insects is not strictly permissible owing to riation in the numbers of larvae used in each experiment.

The detection of proteinases was undertaken by the photographic gelatin ethod described by Parkin (1936).

* All prepared from oak by Wood Chemistry Section, Forest Products Research Laboratory.

RESULTS

Considerable dilution of the enzymes occurs during extraction as describ above, and the clear-cut results of their action which were generally obtained duri the investigation may be taken as evidence of the great activity of these substanc at their normal concentration in the gut. The poor hydrolysis of α-cellulose in t tests may be due to the probable increase in resistance to hydrolysis of cellulo when submitted to the action of strong reagents during isolation from wood. should also be noted that the hemicellulose A used in the tests was extracted fro oak sapwood and contained some 10 % of glucose residues, whereas the hem cellulose B was prepared from heart-wood and was free from hexosan.

Table I shows the results of the experiments to detect certain enzymes in t digestive tracts of a number of species of wood-boring beetle larvae. When sufficie insects were available, results were confirmed by repetition of the tests. The activi

Table I. *The action upon various substrates of the digestive juices of certain wood-boring beetle larvae*

Insect species	Substrate						
	Starch	Sucrose	Maltose	Lactose	Cellulose	Hemi-cellulose A	Hemi-cellulose B
SCOLYTIDAE							
Phloeosinus bicolor	++	++++	+++	+++	o	+++	++++
CERAMBYCIDAE							
Rhagium mordax	++++	++++	o	+++	+	++++	++++
Phymatodes testaceus	++++	++++	+++	+++	++	+++	++++
Isotomus speciosus	+++	++++	+++	+++	+	++++	
Xylotrechus rusticus	++++	++++	o	o	+	+++	+++
Hylotrupes bajulus	+++	+	++	++	+	++	++
Smodicum cucujiforme	o	o	o	++	+	++++	
LYCTIDAE							
Lyctus brunneus	+++	+++	+++	+++	o	o	++
BOSTRYCHIDAE							
Heterobostrychus brunneus *Bostrychoplites cornutus*	++++	++++	+++	+	o	o	o
ANOBIIDAE							
Anobium punctatum	++	+	+	++	+	++++	++++
Xestobium rufovillosum	+++	+++	+++	+++	+	++++	+++
Ernobius mollis	+++	+++	+	+	+	++	++++
Ptilinus pectinicornis	++	++	++	+	+	+++	+++

of the enzymes sometimes varied slightly in different batches of larvae of the sam species and the value included in the table represents the highest recorded.

For the sake of convenience the results of this investigation together with thc of other investigators will be considered family by family.

SCOLYTIDAE

Beetles of the family Scolytidae may be classified in two groups of entire different habits according to whether the life cycle is passed in the solid wo

Ambrosia beetles) or in the bark (bark beetles). Members of the first group are ependent for nourishment upon fungi growing on the walls of their galleries but othing is known of the physiology of their digestion. In the second group the nly species upon which information is available is the ash bark beetle, *Hylesinus raxini* Panz. Hopf (1938) has shown by tests for digestive enzymes that the larvae robably feed upon soluble sugars, starch, the hexosan part of the hemicelluloses, nd proteins. These results he confirmed, so far as possible, by chemical analysis f the food and frass, assuming, in the absence of a positive result in his tests for cellulase, that cellulose is not hydrolysed. He reports the absence of an enzyme apable of hydrolysing the hemicellulose A of ash bark and claims that, so far as emicelluloses are concerned, the larvae are unable to break down the pentosan nd can utilize only the hexosan part of hemicellulose B.

The detection during the present work of amylase, invertase, maltase, lactase, nd proteinase in *Phloeosinus bicolor*, together with a negative result for cellulase, grees with the findings of Hopf. However, an enzyme which strongly hydrolysed emicellulose A prepared from oak sapwood was also found, and since, according o O'Dwyer (1937), this hemicellulose contains only 9–10 % of anhydroglucose esidues, a considerable portion of the pentosan fraction must also have been ydrolysed. The utilization of pentosans is confirmed by the very strong action f the digestive juice upon hemicellulose B which was prepared from oak heart-ood and contained no starch or other hexosan.

No mention is made in the table of *Scolytus destructor*, since the result of tests ith a tissue suspension of the larval guts was entirely negative. This is extremely teresting, as the larvae were fully grown and hibernating: many of the larvae upated after being kept in a warm room for three days. Wood was not present in ie larval guts nor was there any appreciable quantity of digestive juice, and it may nerefore be concluded that, at the start of the diapause with consequent cessation f feeding, the larvae had ceased to secrete digestive enzymes.

Cerambycidae

The large size of the larvae has probably induced several workers to con-ntrate on this family. Seillière (1905) detected an enzyme hydrolysing xylan in ie alimentary canal of *Phymatodes variabilis* L. Falck (1930*a*) and Horn (1930) ave analysed the food and frass of *Hylotrupes bajulus* L. and shown that during igestion depletion of cellulose and pentosans occurs. More recently, Schuch (1937) as shown that some constituent of the cell contents is necessary for the best rowth of the larvae of this insect, since they increase in weight much more rapidly hen feeding in the outer zone of the sapwood than in the inner zone or in heart-ood. In 1938, Becker observed a considerable acceleration of growth when larvae ere inserted into wood impregnated with peptone solution, but not when into ood impregnated with soluble starch or glucose: an intermediate value was btained with an extract of malt. He suggests that the amount of protein in wood ay be a governing factor in the growth of the larvae and that a small amount of trogenous matter was present in the malt extract. Far more striking, however,

are the results reported by Gösswald (1939) who found that, 180 days after hatchin larvae in wood impregnated with 5 % diastase solution weighed up to 114 mg while similar larvae in peptone-wood weighed up to 4 mg. and in the untreate controls up to 2 mg. No explanation is offered for this phenomenon. It seems th it cannot depend upon the enzymic action of diastase on the wood starch, since th quantity present in softwood timbers is very small, but it is possible that the diasta contains as an impurity some growth-promoting substance the presence of whic enables the larva to utilize larger amounts of some or all of the major componen of the wood.

Ripper (1930) has demonstrated the presence of a cellulase in the mid-gu fluid of larvae of *Cerambyx cerdo* L., *Rhagium bifasciatum* F., and *Leptura* sp. I 1934 Müller showed by chemical analysis that there is a considerable loss in cellulo and pentosans from wood passing through the larval gut of *Oxymirus cursor* L *Leptura rubra* L. and *Gracilia minuta* F. and also detected in the alimentary can of *Oxymirus cursor* the enzymes cellulase, hemicellulase, xylanase, amylase, inve tase, maltase, emulsin, lipase, trypsin and erepsin. Mansour & Mansour-Be (1934*a*, 1937) have shown also that the larvae of *Macrotoma palmata* F. an *Stromatium fulvum* Vill. possess an enzyme hydrolysing cellulose, but in the fir paper record the discovery of a Cerambycid larva, *Xystrocera globosa* Ol., whic cannot secrete a cellulase and appears to derive its food from the minor carbohydra constituents which occur in plenty in the sapwood in which it lives. Finall Pochon (1939) has isolated a cellulolytic bacterium from the larval digestive tra of *Rhagium sycophanta* Sch. living in rotten wood and states that it is probab responsible for the digestion of cellulose in this species, but this assumption open to the criticism that the bacteria may perhaps occur normally in decayin wood and are merely incidental in the gut of the insect.

As shown in Table I, the results of tests upon six species of Cerambycid larv proved very uniform. The only comparable investigation, made by Müller (193 upon the larvae of *Oxymirus cursor*, gave results in close accord with those of th author. In general, therefore, it may be concluded that the majority of Cerambyc larvae digest the starch, soluble sugars, hemicelluloses, cellulose and proteins wood.

It is noteworthy that there is no fundamental difference between the digesti powers of species feeding upon bark, phloem and cambium, e.g. *Rhagium*, *Phym todes*, and those living in the solid wood, e.g. *Isotomus*, *Xylotrechus*, *Hylotrup Smodicum*, although, according to the one test undertaken, larvae of the last name insect seem to be deficient in enzymes hydrolysing the cell content carbohydrate

The number of genera of this family in which a cellulase has been reporte now amounts to twelve, the only exception so far recorded being *Xystroce globosa* which, according to Mansour & Mansour-Bek (1934*a*), lacks a cellula and is dependent for its food upon cell contents.

Lyctidae

Mer as early as 1893 intimated that starch in timber was the principal food of *Lyctus* larvae. In 1929, Campbell reported that chemical analyses of food and frass showed no difference in the proportions of the major components and concluded that the larvae must derive their nourishment solely from the cell contents. Wilson (1933) has since confirmed Mer's statement that *Lyctus* cannot develop in starch-free wood. Parkin (1936) concluded that the larvae require starch, protein and an unidentified water-soluble substance, and also succeeded in rearing young larvae to beetles upon an artificial diet of starch, sucrose and peptone in the complete absence of wood. In addition, he detected in the larval gut the ferments amylase, invertase, maltase, lactase and proteinase. The presence of these enzymes and the absence of any capable of hydrolysing cellulose or hemicellulose A have been confirmed during the present series of tests. A new result, however, is that the digestive fluid of the larvae can cause a partial hydrolysis of hemicellulose B, but whether this can be done when the substrate is incorporated in the structure of the cell wall requires further investigation.

The ease of detection of starch in wood by means of a dilute aqueous iodine solution makes it possible to obtain some additional information on the action of the amylase. Microscopical examination shows the wood particles in the mid-gut of *Lyctus* larvae to be so small that nearly every cell is broken open, yet starch grains can often be found in the rectal contents and in the bore-dust in the tunnels. There is always less starch in the frass than in the original wood but the percentage reduction is very variable, suggesting that the activity of the amylase differs in different larvae. In abundantly starchy wood, *Lyctus* bore-dust sometimes contains a considerable quantity of starch and, when tunnels cross, less starch is often detectable at the point of intersection, indicating that further nourishment is obtainable from the starchy frass when eaten by a second larva.

Bostrychidae

Little is known of the food requirements of this family, closely related to the Lyctidae, but Beeson & Bhatia (1937) in their recent summary of knowledge on the biology of the Indian species state that starch is an essential constituent of the food of *Dinoderus*, *Heterobostrychus* and *Sinoxylon*, if not of all Bostrychidae. With reference to attack by *Dinoderus* spp. in bamboo, they summarize the results so far obtained as follows:

"An attempt to reconcile the data and explain the contradictions and inconsistencies of the experimental work... is foredoomed to failure; but one is left with the impression that (*a*) starch is an essential food-substance, (*b*) glucose is not essential, (*c*) a soluble substance possibly a disaccharide is an essential, and that (*d*) neither of the essentials alone is sufficient."

The results of the present investigation show that the larvae of *Heterobostrychus* and *Bostrychoplites* feed exclusively upon the cell contents, namely starch, sugars

and proteins. Unlike *Lyctus* larvae they are apparently unable to utilize any portion of hemicellulose B.

ANOBIIDAE

According to chemical analyses of wood and frass by Falck (1930*b*) and Müller (1934), *Anobium punctatum* larvae can utilize cellulose and hemicellulose. In the case of *Xestobium rufovillosum*, Campbell (1929) reported, after analysing sound oak heartwood and larval frass, that the larvae of this beetle bring about a change in the carbohydrate:lignin ratio of oak which indicates utilization of the cell wall carbohydrates. Similar analyses have been performed by Ripper (1930) and Norman (1936). None of these authors has taken into consideration in the interpretation of his results the change in composition of wood brought about by fungal decay, which has been shown by Fisher (1935) to be a necessary preliminary to *Xestobium* attack. Ripper, however, confirmed his conclusion that cellulose was digested by detecting the presence of a cellulase in the larval gut fluid.

Four members of this family have been investigated and a positive result obtained for each enzyme test undertaken. Therefore the larvae of *Anobium*, *Xestobium*, *Ernobius* and *Ptilinus* are able to break down the carbohydrates of the cell wall, namely cellulose and hemicelluloses, as well as to utilize the protein and, when they are present, starch and sugars in the cell contents.

DISCUSSION

On the basis of their nutrition wood-eating insects have been divided by Mansour & Mansour-Bek (1934*a*) into two types, namely those which have no cellulase and can consequently live only in timber comparatively rich in starch and sugars, and those able to secrete a cellulase and therefore to live in timber relatively poor in these cell contents. The results of the present work indicate, however, that there are three groups into which the wood-boring Coleoptera may be divided on the basis of their digestive powers as follows:

(i) Larvae able to utilize only the cell-contents and perhaps part of the polysaccharides which are intermediate in composition between starch and the hemicelluloses—Lyctidae and Bostrychidae.

(ii) Larvae able to utilize cell contents and the carbohydrates of the cell wall up to hemicelluloses, but excluding cellulose—Scolytidae (bark beetles).

(iii) Larvae able to utilize cell contents and the carbohydrates of the cell wall including cellulose—Anobiidae and most Cerambycidae.

The position of the Cerambycid, *Xystrocera globosa*, which is reported by Mansour & Mansour-Bek (1934*a*) to lack a cellulase, is not clear as these workers did not determine whether the larva could digest hemicelluloses.

It is interesting, for the purposes of comparison, to note that at least two types of fungal attack occur in wood. Most of the surface moulds appear to live solely upon cell contents and their hyphae pass from cell to cell through natural openings such as pores. In the wood-rotting Polyporaceae, twenty-six species of which have

been investigated by Bose & Sardar (1937) and Garren (1938), ferments causing the breakdown of cellulose, hemicellulose and lignin are of fairly general occurrence. These Basidiomycetes extract nourishment from the cell wall and pass from cell to cell by perforating the wall itself through enzymic action.

There can now be no doubt of the existence of a cellulase among the digestive enzymes of many wood-boring beetle larvae, although it is not of universal occurrence. The view advanced by several authors that the digestion of cellulose by coleopterous larvae is dependent upon a symbiosis with micro-organisms which are responsible for secretion of cellulase has now been shown by Müller (1934) and Mansour & Mansour-Bek (1934*a*) to be untrue, at least with reference to the Cerambycidae and Anobiidae. However, in the light of Koch's (1934) work on *Sitodrepa panicea* and similar research on other insects which are not wood-borers, it is possible that the organisms are important in connexion with the vitamin requirements of the insects.

According to Fraenkel (1936) and others, the extent of utilization of pentose sugars varies considerably among insects but, in view of the strong action of their digestive juices upon the hexosan-free hemicellulose B of oak heartwood, it must be accepted that the larvae of many wood-boring Coleoptera can use them in their metabolism.

The source of nitrogen for insects feeding on wood has long been a subject of discussion, since some workers have thought that wood was lacking in protein. Heitz (1927) suggested that the internal symbionts might be able to fix atmospheric nitrogen, but Müller (1934) has been unable to find any evidence to support this as a result of artificial culture of the organism. Ripper (1930) and Mansour & Mansour-Bek (1934*b*) assume that insects can derive their nitrogen supply directly from the wood, but no attempt had been made to determine whether proteolytic enzymes occur in the alimentary canals of wood-boring insects until Parkin reported in 1936 the discovery of a strong proteinase in the digestive juice of *Lyctus* larvae. At the same time he pointed out that in wood attacked by *Lyctus* the carbohydrate:protein ratio of the cell contents is such that the larvae might be expected to obtain their supply of nitrogen from the wood without difficulty. Hopf (1937) has since shown that the larvae of *Hylesinus fraxini*, *Lyctus* sp., and *Anobium punctatum* cause a reduction in protein nitrogen of the wood as it passes through their intestines and has detected (1938) trypsin and erepsin in larvae of *Hylesinus*. The present demonstration of a proteinase in the larval guts of fourteen species of wood-boring Coleoptera belonging to five families indicates that such a ferment occurs generally and that the larvae are not dependent upon symbiotic micro-organisms for their nitrogen supply.

According to the results of this investigation there would seem to be no reason why some of the species examined should not be capable of deriving nourishment from wood known to be unsuitable. For instance, *Hylotrupes bajulus* confines its attack to coniferous timbers and is unknown in hardwoods although the enzymes present in the larval gut would apparently fit it to digest either kind of wood with equal facility. Similarly, many borers are recorded only in hardwoods and some

insects are confined to one or very few species of timber. Furthermore, no explan tion has yet been found for the restriction of certain insects, e.g. *Anobium pun tatum*, to seasoned wood when it might be supposed that they could feed equall well in unseasoned wood and vice versa. It is clear therefore that the nutrition wood-boring Coleoptera depends upon more factors than the enzyme complex the larval gut and much research remains to be carried out before any compr hensive idea can be gained of the interaction of the various conditions governin the food relationships of wood-eating insects.

SUMMARY

Qualitative tests for the presence of amylase, invertase, maltase, lactase, cell lase, hemicellulases A and B, and proteinase in the digestive juices of wood-borin coleopterous larvae are described.

Fourteen species representing the families Scolytidae, Cerambycidae, Lyctida Bostrychidae and Anobiidae, have been investigated and their nutrition is discusse in terms of the enzymes found and present knowledge of the composition of woo

It is concluded that three types of wood feeding may be distinguished, (i) larv able to utilize only the cell contents and perhaps part of the polysaccharides whi are intermediate in composition between starch and the hemicelluloses—Lyctid and Bostrychidae, (ii) larvae able to utilize cell contents and the carbohydrates the cell wall up to hemicelluloses, but excluding cellulose—Scolytidae (ba beetles), and (iii) larvae able to utilize cell contents and the carbohydrates of the ce wall including cellulose—Anobiidae and most Cerambycidae.

A proteinase is of general occurrence in the larval guts of wood-boring beetle

Digestive enzymes were absent from the gut of hibernating larvae of *Scolyt destructor*.

The work described above was carried out as part of the programme of th Forest Products Research Board and is published by permission of the Departme of Scientific and Industrial Research. The author wishes to thank Dr R. C. Fishe officer in charge of the Entomology Section, and Mr W. G. Campbell, officer charge of the Wood Chemistry Section, for their helpful criticism of the manu script.

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PHYSIOLOGICAL EFFECTS OF A HYPOTONIC ENVIRONMENT

II. SHOCK EFFECTS AND ACCOMMODATION IN CILIA (*PLEUROBRACHIA, MYTILUS, ARENICOLA*), FOLLOWING SUDDEN SALINITY CHANGE

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(*Received* 16 *May* 1940)

(With One Text-figure)

If polychaete rhythmic muscles are suspended in sea water, which is then suddenly replaced by dilute sea water, they typically respond to the change by showing, first excitation, then inhibition, then accommodation to the new fluid (Wells & Ledingham, 1940*a*). Shock effects, followed by accommodation, have also been shown to follow sudden salinity change in marine amoebae (Pantin, 1924) and in the hearts of Limulus, Carcinus and a tortoise (Carlson, 1905; Girault, 1935). The experiments here described were undertaken primarily in order to find out whether similar phenomena occur in cilia. The action of hypotonic fluids on the lamellibranch gill has been described by Fredericq (1922) and by Tomita (1934), but neither author paid any attention to the behaviour of the cilia immediately after salinity change.

Some experiments on the rate of swelling of Mytilus gills were also carried out in the hope of throwing light on the mechanism of accommodation.

COMB-PLATES OF *PLEUROBRACHIA PILEUS*

In this ctenophore, as is well known, the locomotor cilia (to which we confined our attention) form strong comb-plates arranged in eight symmetrically spaced longitudinal rows along the animal's surface. Individuals about a centimetre long were caught from Bangor pier and studied in the laboratory at 11–14° C.

If a whole Pleurobrachia is suddenly transferred to 40% sea water,[1] the comb-plates at once stop beating, stand out at right angles to the animal's surface, and become incoherent and ragged. The individual filaments composing the plate seem to swell almost instantaneously. They become gelatinous in appearance, and less definite in outline.

In 2 or 3 min., slight flickering movements, at first intermittent and then continuous, appear here and there among the comb-plates. The number of active points steadily increases. This resumption of movement occurs irregularly over the

[1] To avoid *p*H changes, all dilutions were made with well-aerated $M/400$ $NaHCO_3$.

nimal's surface, suggesting that the different individual elements vary in sensitivity nd each recovers independently of the others. Thus, one row of combs may be uiet while the next is briskly vibrating; or a comb may be beating while its neighours on each side are still motionless; or part only of a comb may be active. After or 3 hr., most of the cilia are quite vigorously active and often individual rows how correlated movement of the combs. But even after three hours, the movement s still of an abnormal type. The combs are still rather ragged and tend to stick out at ight angles to the surface, and their activity consists of a rapid vibration, of high requency and small amplitude. The animal as a whole does not move along.

Meanwhile the body (and especially the lips) swells; the tentacles are kept reracted; and after an hour or so, the surface membrane of the ctenophore is raised nto a number of small blisters.

On returning to sea water, after 3 hr. in 40%, ciliary activity again ceases intantly. The plates project stiffly and raggedly once again. The filaments composing he plates lose their swollen appearance and become fine and silky, practically intantaneously. Slight flickering appears, here and there, after 2 or 3 min., and the ctivity slowly spreads, in an irregular way, over the whole surface. The general icture of inhibition and accommodation is essentially the same for the downward nd for the upward change. In the latter case, however, the type of beat and the ttitude of the cilia gradually become more and more normal, after passing through phase of rapid flickering like that seen in the dilute water.

. The ultimate extent of recovery in sea water, after 3 hr. in 40%, is surprisingly omplete, in spite of the very miserable appearance presented by the ctenophores in heir abnormal environment. After from 2 to 4 hr. in sea water, the locomotor cilia eat vigorously and normally, with metachronal correlation of the plates; the animal s a whole swims along; and it is possible, by prodding it, to witness the usual eflex changes in the activity of the locomotor apparatus.

The effects described above are undoubtedly due to direct action of the chemical nvironment on the effector cells. Similar results can be got with isolated rows of omb plates, freed as far as possible from jelly, or even with single comb plates (each vith a fragment of tissue attached), made by cutting up the isolated rows. In all ases, inhibition and accommodation follow the changes from 100 to 40% and back gain. The dissected preparations give somewhat longer inhibition periods than do he intact animals; this is probably because the jelly, in the whole animals, "damps" he salinity change to some extent before it acts on the ciliated cells.

DIRECT OBSERVATION OF *MYTILUS* CILIA

These experiments were made at 10–15° C., on the gill filaments of animals ollected at Bangor. The filaments were mounted in a cell so constructed that the ilia could be watched (with a $\frac{1}{3}$ in. objective) while one fluid was replaced by nother.

The different types of cilia show quantitatively different reactions. If attention s focussed on a portion somewhere near the middle of a filament, the following

types are easy to watch: The latero-frontals; the frontals; the short abfrontals (on the opposite side of the filament); and the long abfrontals (seen here and there among the short abfrontals).

On suddenly replacing sea water by 40% sea water, the following changes are seen. The amplitude of beat of the latero-frontals begins to fall at once and in about 30 sec. they have stopped in the "extended" position. Meanwhile the amplitude of the frontals also falls off, until in about a minute they show only a very small trembling movement. However, in most cases at least, the frontals do not stop altogether in 40%, as the latero-frontals do. Both types then accommodate themselves. One or two latero-frontals, scattered at random along the row, begin to show feeble movements 5 or 6 min. after the change; the number of active cilia and the vigour of those that are active, then steadily increase during the next 10 or 15 min., at the end of which time all the latero-frontals are beating quite vigorously. The frontals also revive gradually, but somewhat more rapidly than the latero-frontals. A sharp contrast to the cilia of the frontal face is afforded by the short abfrontals, which are apparently quite unaffected by the change, and show no detectable loss of vigour at any time. The long abfrontals are however temporarily inhibited. The improvement steadily continues, and in a few hours the filament seems to be in excellent condition.

The reverse change—40–100%—is rather variable in effect. It usually causes some degree of inhibition in the latero-frontals, which may be obviously slowed or may even stop altogether for half a minute or so. In most cases at least, inhibition is not seen in the frontal cilia; they are however stopped for about half a minute on returning from 25 to 100%.

It is possible, then, by changing from 100 to 40%, to show "shock effects" in the cilia similar to those seen in Pleurobrachia and in polychaete muscle. Additional observations were made with other salinities, some with the observation cell mentioned above, and others by simply transferring filaments from one watch-glass of fluid to another. The following points emerged:

(1) The different types of cilia vary greatly in sensitivity. That the frontals are less sensitive than the latero-frontals has already been seen, for the inhibition produced by 40% is shorter and less complete in the former than in the latter. On suddenly changing from 100 to a still lower salinity, the frontals are completely inhibited, but always for a less time than the latero-frontals. With 30% sea water, for example, the frontals are inhibited for 6–16 min. and the latero-frontals for 1–3 hr. The most resistant cilia are those of the food groove at the end of the filament, and the short abfrontals. The long abfrontals, on the other hand, are at least as sensitive as the latero-frontals.

(2) The short abfrontal cilia presented another feature of considerable interest. As already noted, these cilia are very resistant; a change from 100 to 25% only produces a slight temporary reduction in amplitude. With 20 or 15%, they show a double shock effect—there is first a phase of definite acceleration, lasting for about a minute; then activity gradually falls off and the cilia become completely quiet. Finally, in 15–20 min., the usual slow recovery occurs.

(3) At salinities below about 35 %, marked disintegration of the gill sets in. Cells can be seen to swell greatly and escape from the epithelium, often with their cilia still beating. At 30 % whole patches of the gill surface may slowly denude themselves by this means. It seems from our experiments that the salinity range over which the gill can function usefully is more likely to be limited by mechanical effects of this kind than by direct osmotic actions on the actual process of ciliary movement. According to Conklin & Krogh (1938) however, *Mytilus* penetrates into the bay of Bothnia where the salinity is 4·5–5 ‰, or about 15 % of sea water. We must therefore infer, either that the Baltic animals are physiologically different from the Bangor ones, or that the Bangor animals would not have shown disintegration if the change had been made in some other way—e.g. very slowly.

QUANTITATIVE EXPERIMENTS ON THE *MYTILUS* GILL

The aim of these experiments was to follow the changes in water content of the cells resulting from sudden dilution of the medium, and to compare them with the mechanical responses. The work was done in London, at 15–16° C., on Plymouth mussels.

Mechanical activity was measured by timing the rate at which a standard platinum weight crossed the gill. Each point in Fig. 1 represents the mean of ten readings, and is expressed as percentage of the mean of 20 measurements previously made in sea water.

Water content was determined by making wet and dry weight measurements on pieces of gill which had been exposed to the solutions for various lengths of time. Each point in Fig. 1 is a single determination.

Swelling, as the figure shows, is very rapid. More than half the water is taken up in the first 2 min. Mechanical activity does not reach its final value until some time after swelling is complete. This is particularly clear in 30 % sea water, in which case it seems likely that the gill has already reached osmotic equilibrium during the period of total inhibition of the cilia. The mechanical activity measured is mainly that of the frontal cilia, while the water content is that of the whole gill. It is improbable that the different types of cell swell at very different rates; and in the latero-frontal cells, whose cilia show much longer inhibition than the frontals, the contrast between rate of swelling and rate of mechanical recovery is presumably even greater than that shown in Fig. 1.

We conclude that sudden transfer of a gill to hypotonic sea water is followed by two distinct events: (1) rapid water uptake, accompanied by cessation of mechanical activity in most types of cells; (2) subsequent accommodation, a process which does not involve further changes in the water content of the cells.

The effect on mechanical activity of sudden return from dilute to normal sea water was also studied. Activity increases about up to the original sea-water value, but does not pass above it (i.e., there is no excitation, like that seen in the *Arenicola* extrovert; see Wells & Ledingham, 1940 *a*, Fig. 7). As is to be expected from the results of direct observation of the cilia, the activity measurements often show a

short decrease in activity immediately after an upward change; this inhibition i however usually too small and quick to be demonstrated effectively by the quanti tative method.

NEPHRIDIAL CILIA OF *ARENICOLA MARINA*

As the salinity relations of a rhythmic muscle preparation from this species hav been described in detail elsewhere (Wells & Ledingham, 1940*a*), we thought i worth while to make parallel observations on the nephridial cilia.

The *Arenicola* nephridium consists of (1) a funnel, with a number of finger-lik vascular processes on the dorsal lip; (2) a large, thin-walled excretory sac, into whic

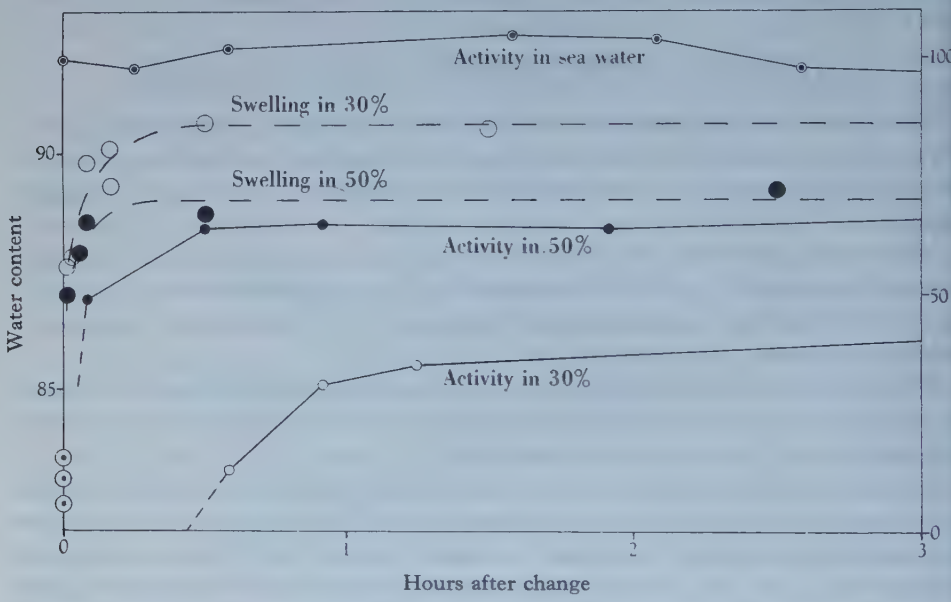

Fig. 1. *Mytilus gill.* Mechanical activity (as percentage of the activity in sea water) and wate content (as percentage of the wet weight) at various times after transfer from sea water to hypotoni fluids. Large circles = water content; small circles = activity. Black circles = in 50 %, white = i 30 %, circles with black dot = in 100 % sea water.

the funnel opens; (3) a muscular vesicle, leading by a short duct to the exterior. Th dorsal lip processes of the funnel are covered with small but very active cilia. Th excretory sac is lined with long cilia, which are particularly conspicuous in the regio near the funnel opening.

Bangor animals were used. The nephridia were excised, split up along the lengt of the excretory sac, and observed with a $\frac{2}{3}$ in. objective.

The results were like those obtained on Lamellibranch and Ctenophore cilia Sudden change from 100 to 50 % sea water was not sufficient to cause shock in hibition. On transferring nephridia from 100 to $33\frac{1}{3}$ %, the activity of the cilia fe within 30 sec. to a small rapid flicker and finally, in the case of the dorsal lip cilia, t a complete standstill. In 3–5 min., the inhibition began to pass off, but the cilia di

not regain their full power, estimated by their ability to drive particles along, for 7–8 hr. Return to sea water, after accommodation to 33⅓%, caused stoppage for about a minute.

At salinities below 33⅓%, the following points were noted. Even after accommodation, activity is feeble in 20%, the cilia of the excretory sac being more affected than those of the dorsal lip. Only the latter are active in 15%. Marked disintegration of the tissues also occurs. In 25% the surface gets clogged in many places by swollen and escaping cells, and even in 33⅓% a certain amount of disintegration is seen. *Arenicola marina* occurs in nature at salinities a little below 25%, so we seem to have the same problem here as in the case of *Mytilus* cilia, or of the *Arenicola* extrovert (Wells & Ledingham, 1940*a*). Either our experiments, for some reason, do not give the true "lower limit" down to which the nephridium can function; or there are physiological differences between the individuals used by us and those found in very dilute habitats.

DISCUSSION

There is clearly a general resemblance in salinity relations between cilia and polychaete rhythmic muscles. The most important points are as follows:

(1) A sudden downward change in salinity causes inhibition, followed by accommodation. To cause complete inhibition, the drop must be fairly big, but to allow accommodation, the final salinity must be above the "lower limit" characteristic of the tissue studied. In most cases, a drop from 100 to 30 or 40% sea water serves to demonstrate these effects.

(2) Except in the case of the *Arenicola* extrovert, temporary inhibition also results from a sudden upward change of sufficient magnitude.

(3) In some cases only (polychaete muscles, *Mytilus* short abfrontal cilia), the inhibition evoked by downward change is preceded by a phase of excitation.

At present, few published data are available for comparison. It is a curious fact that, although the effect of varying individual ions (at approximately constant osmotic pressure) has been tried on numerous preparations, and by numerous authors, very little has been done on the results of varying the total concentration while keeping the balance of the individual salts constant. Of the published studies, some are useless for our purpose, for they ignore the importance of the time factor, and either give only the immediate response to a change of osmotic pressure, or describe the result of long exposures without noticing the immediate effects.

In one case, shock effects of sudden salinity change appear not to occur. Crozier (1916) exposed the cloacal complex of a large Bermudan holothurian to various dilutions of sea water, and found that pulsation rate fell steadily with time. This preparation is very bulky, and the muscle fibres are imbedded in a rather gluey connective tissue. Possibly the sharpness of the salinity changes was damped by slow diffusion into the tissue, before they could act on the pacemaker cells.

Bethe (1908) got similar results with the jellyfish *Rhizostoma*. In dilute sea water, pulsation rate fell with time. The evidence on this point is however contradictory, for Fredericq (1922) reports that *Rhizostoma*, suddenly transferred to 46% sea

water, "paraissent malades au début de l'expérience, mais ils se remettent bientôt" after 53 hr., "ils semblent de bonne santé", although considerably swollen. Th nature of their temporary malady is unhappily not specified. A shock effect, followe by accommodation, is perhaps concerned.

In the *Limulus* heart (Carlson, 1905), accommodation to a new salinity occur but in this case hypotonic media have a stimulating action on amplitude and rat "There is a gradual 'adaptation' of the ganglion to the new osmotic conditions... The stimulating action of the diluted plasma or sea water reaching its maximum in few minutes. This maximum is soon followed by a return towards the norm rhythm." Return to sea water, or change from sea water to a hypertonic mediu has the opposite effect, i.e. slowing followed by accommodation. In the same pape Carlson mentions a few experiments on the tortoise auricle, which behaves similarl The crustacean heart appears also to resemble that of *Limulus* in its reactions t salinity change (Girault, 1935).

In certain cases (polychaete muscles, *Mytilus* short abfrontal cilia) sudden dilu tion produces excitation followed by inhibition. This may be a combination of tw actions seen separately in the *Limulus* heart, which is only excited, and the *Pleuro brachia* comb or the *Mytilus* latero-frontal cilium, which are only inhibited, unde these conditions.

Pantin (1924) showed that in marine limax amoebae, sudden salinity chang produced distortion followed by some degree of spontaneous recovery in the ne medium. The abnormalities of form produced by downward and by upward change are quite different, a fact which suggests that *Amoeba* resembles the *Limulus* hea more than it does a cilium or a polychaete muscle in its salinity reactions.

It appears, then, that shock effects of sudden salinity change, followed b accommodation, occur widely among contractile tissues. The details of the shoc effects are however variable.

Our quantitative experiments on the *Mytilus* gill indicate that the accommoda tion process, whatever its intimate nature may be, is not an adjustment of the wate content of the cell. This warns us against assuming that the effects of varying th external osmotic pressure are due to changes in the intracellular water content. It of course by no means inevitable that a physiologically balanced mixture of salts wi remain balanced if diluted. In certain cases, ions act more in virtue of their absolu individual concentrations than in balance with other ions, and even where geo metrical ion antagonisms have been demonstrated, it has sometimes been shown tha they operate only over a quite limited range of absolute concentrations (Jendrassi & Annau, 1925; Clark, 1926; Wells, 1928). On doubling the total concentratio of a saline suitably balanced for the snail's heart, disturbances of rhythm appea which are due to the raised potassium concentration (Cardot, 1921, 1922). More over, changes in the concentrations of individual ions, made at constant osmoti pressure, may result in shock effects followed by recovery of normal behaviou examples of this are the well-known potassium paradox (Libbrecht, 1920, 1921 Jendrassik, 1924; Wells, 1928; Chao, 1934) and the responses to variation i magnesium concentration shown by the *Arenicola* extrovert (Wells & Ledingham

1940 *b*). An apparently osmotic shock effect might therefore be due in reality to the change in concentration in one of the ions. In a word, the problems presented by a hypotonic environment are not as simple as they seem to be at first sight. The results of exposing organs to dilutions of their normal media may be directly applicable from the ecological point of view, but when one comes to the problem of their physiological mechanism, one must watch for the possibility of individual ion actions masquerading as osmotic pressure actions.

SUMMARY

1. The cilia of *Pleurobrachia*, *Mytilus* and *Arenicola* show inhibition, followed by accommodation, as a result of sudden downward or upward changes in the salinity of the bathing medium.

2. Variations in sensitivity occur between different species, and between different types of cilia in the same species.

3. When *Mytilus* gills are suddenly transferred from 100 to 30% sea water, they take up water very rapidly. Accommodation, as indicated by the mechanical activity, continues long after osmotic equilibrium has been reached. Therefore, accommodation is not an adjustment of the water content of the cell.

4. The results are discussed, and compared with those obtained on other types of contractile tissues.

The work was done, partly at Bangor (by G. P. W. and I. C. L.), and partly at Birkbeck College, London (by M. G.). Our thanks are due to Prof. Brambell and Prof. Jackson for allowing us to work in their Departments.

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THE PHYSIOLOGICAL ACTION OF ABNORMALLY HIGH TEMPERATURES ON POIKILOTHERM ANIMALS[1]

II. THE RESPIRATION AT HIGH SUBLETHAL AND LETHAL TEMPERATURES

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(*Received* 26 *May* 1940)

(With Four Text-figures)

INTRODUCTION

Most warm-blooded animals and many insects are killed if their body temperature is held at about 40° C. for any length of time, and for many other cold-blooded animals even lower body temperatures are fatal.

There has been a considerable amount of speculation as to the causes of death at these temperatures. While it was once commonly assumed that coagulation of proteins was the ultimate cause of death, this theory is now generally abandoned in face of the many records of upper lethal temperatures which are far below the region in which such coagulation of proteins occurs. Various theories have been put forward for explaining death at moderately high temperatures, and a comprehensive review of the situation has been made by Belehradek (1935). One of the better known is the asphyxiation theory of Winterstein (1902, 1905).

It is known that the oxygen uptake of a cold-blooded animal increases with rising temperature up to a certain maximum which is very near the thermal death point. Beyond this maximum the oxygen uptake actually decreases for a very short range of rising temperatures, which indicates that the temperature is producing adverse effects. Winterstein argues that if the temperature increases beyond a certain point the oxygen requirements of the tissues are so high that the oxygen supply becomes inadequate and that death results from the consequent accumulation of metabolites.

If this hypothesis is correct one should expect that raising the oxygen tension in the surrounding medium would prolong the upward trend of the temperature-oxygen-uptake curve beyond the maximum for the normal oxygen tension and would increase the resistance of an organism to a given temperature. It has been the purpose of the present investigation to test Winterstein's asphyxiation theory by studying the respiration of an organism at high (sublethal and lethal) temperatures at different partial pressures of oxygen.

[1] First paper of this series: Fraenkel G. S. & Hopf, H. S. (1940) *Biochem. J.* **34**, 1085.

METHODS

All the experiments on respiration were performed on the modified Barcroft manometer described by Dixon (1934, p. 8). The flasks used have been described in a previous paper (Fraenkel & Herford, 1938). The experiments were carried out with larvae of the blowfly, *Calliphora erythrocephala* Meigen which served as experimental animals in the previous investigation. The respiration was measured at 42° C., which was found to be the temperature at which each determination could be finished within 3 hr. The equilibration period had to be cut short to 15 min. since the influence of the high temperature on the oxygen uptake could be expected to be shown very rapidly. It is possible that in some cases the first reading, covering the second quarter of an hour, is not correct owing to the short equilibration period, but from the second reading onwards the equilibration can be regarded as satisfactory. The equilibration period is not included in our graphs.

In order to obtain determinations which are as far as possible comparable amongst themselves, larvae which had been reared together in one batch were used for four determinations at four different partial pressures of oxygen, with duplicates made in most cases. Each of the Figs. 1–4 represent a group of determinations carried out on material from one batch, a different batch being used for each group. Larvae grown at different periods vary slightly with regard to average size, water and fat content, and since the oxygen uptake shows a considerable range of variation even at normal temperatures (Fraenkel & Herford, 1938) it was hoped to obviate this variation as far as possible by only comparing readings obtained from larvae from the same batch.

Usually five to ten larvae (400–800 mg.) were used for each determination. All results are expressed as c.c. oxygen uptake/g. live weight/hr. at normal temperature and are not reduced to normal pressure.

EXPERIMENTS

The results are represented in Figs. 1–4. Fig. 1 shows that the initial oxygen uptake is entirely dependent on the oxygen partial pressure outside the body. However, even the highest individual reading (0·725 c.c. O_2/g./hr.) for the second 15 min. at 80 % O_2 is still considerably below the average value (0·9 c.c.) obtained previously (Fraenkel & Herford, 1938) for *Calliphora* larvae at 27° C. This indicates that by the time that the first reading was taken—30 min. after the beginning of the experiment—the oxygen consumption has decreased as a result of heat injury. The starting-point of all the curves must therefore be considered to be already on the down gradient

The oxygen uptake at 80 % O_2 partial pressure decreases very rapidly to a very low level. The curve taken at 20 % O_2 (air) starts at a lower level than the 80 % curve, declines at first less rapidly, and from about 45 min. after the start of the experiment is almost identical with the 80 % curve. The 10 % curve starts at a still lower level, but falls off much less rapidly than the 80 and 20 % curves, which it actually crosses after about $\frac{1}{2}$ hr. From then the O_2 consumption in 10 % O_2

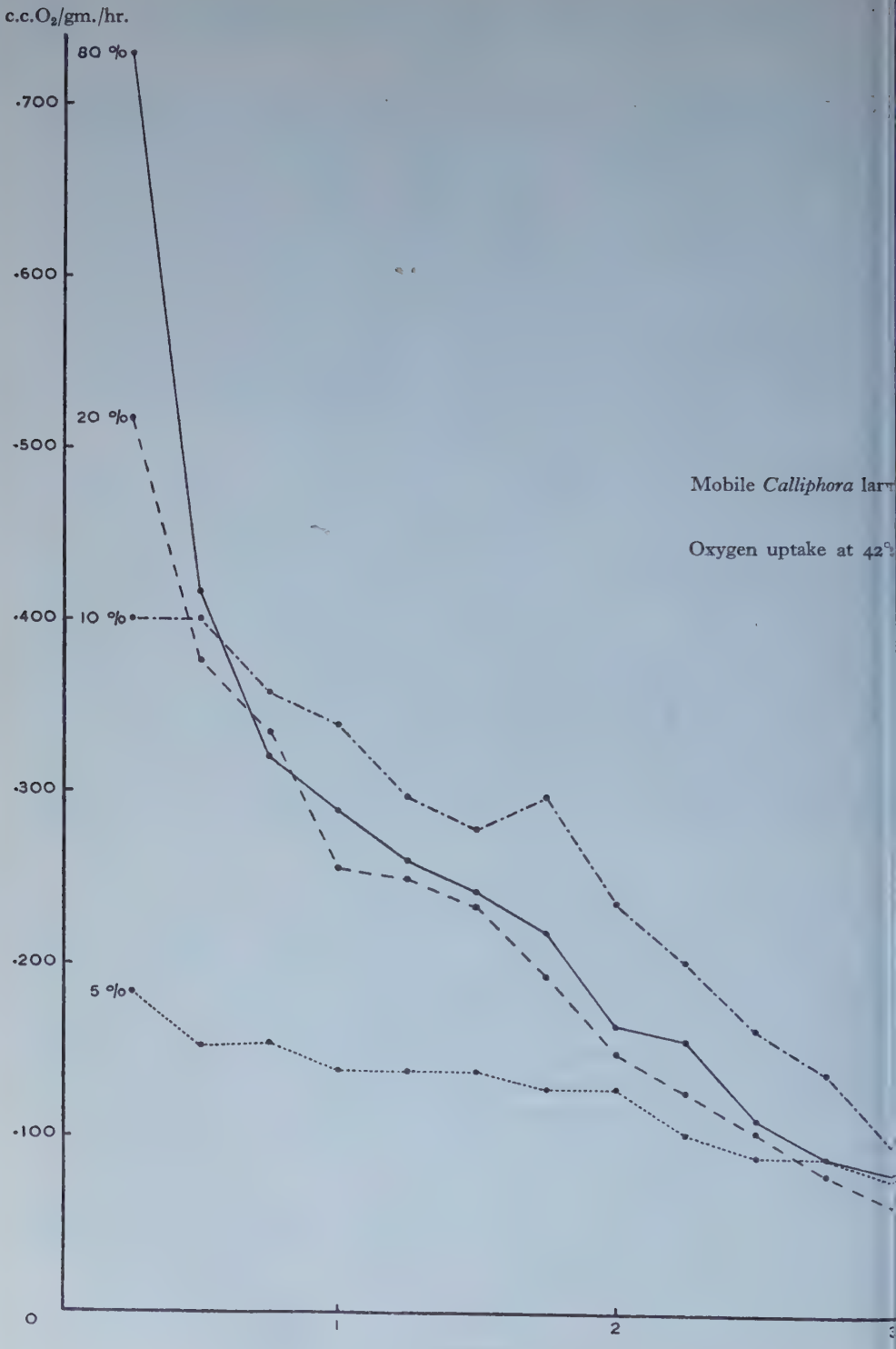

Fig. 1. The oxygen consumption of mobile larvae of *Calliphora erythrocephala* at 42° C. during a period of 3 hr. at different oxygen tensions.

considerably higher than that at 20 and 80 % oxygen. The oxygen uptake at % O_2 starts at a very low level and sinks slowly. After 3 hr. at 42° C. the oxygen onsumption is about the same at the four ifferent oxygen concentrations, which is ot surprising since the larvae are now ead.

In another series of determinations Fig. 2) at 100 % O_2 (1·37 c.c. O_2/g./hr.) he rate of respiration is very high at first ut declines again sharply and becomes fter 45 min. about the same as that in air. The 5 % curve is again at a very low level.

It is a well-known phenomenon in he measuring of the respiration of cold-looded animals that the oxygen consumption starts comparatively high and later eclines. This is due to the fact that animals re stimulated at first to move about after eing placed into a respiratory chamber nd later "settle down". It was important o exclude voluntary body movements in rder to find out to what extent the slope f the curve is influenced by this effect. art of the falling off of the rate of respiration is certainly due to the decrease n muscular activity. It is, however, not ertain to what extent the cellular respiration is affected by the high temperature. or this reason experiments were designed n which only the basal metabolism would e determined. This was done by ligaturing ff and removing the front end of the larvae which contains the whole of the central ervous system (cf. Fraenkel & Herford, 938, Fig. 2 and p. 268). A ligature behind he "ganglion" completely immobilizes he posterior part which, however, remains live and otherwise normal for many lays.

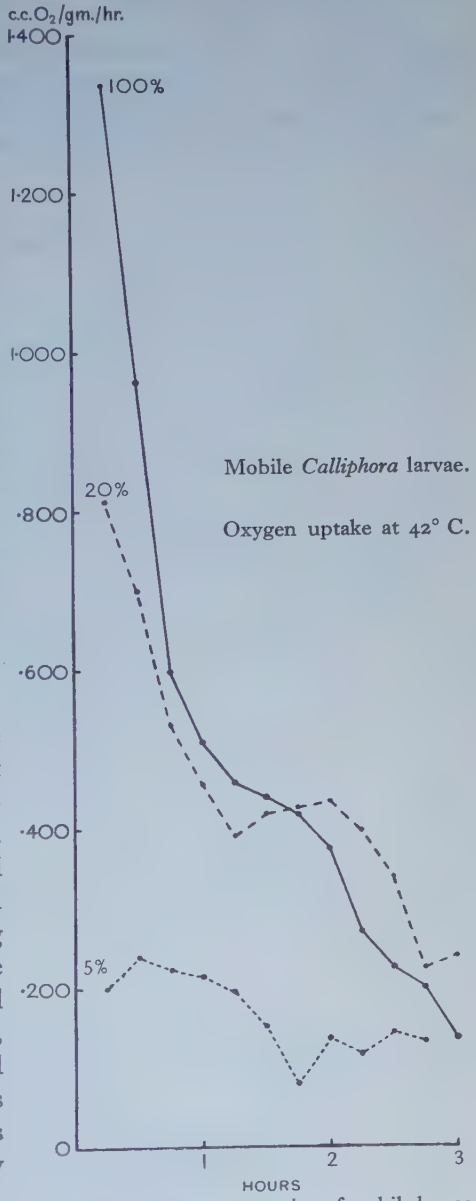

Fig. 2. The oxygen consumption of mobile larvae of *Calliphora erythrocephala* at 42° C. during a period of 3 hr. at different oxygen tensions.

Fig. 3 shows that under these conditions the oxygen uptake starts at a much lower level than in freely mobile larvae (Figs. 1, 2) and that the curves maintain a constant level for at least 1 hr. This suggests that for a considerable period the falling off of the oxygen uptake of mobile larvae simply expresses decreasing

muscular activity which probably partly consists of the "settling-down effect" a partly of an increasing paralysis.

In 100 % O_2 after 1 hr. the curve falls sharply which suggests that the cellu respiration now becomes seriously affected and the animal is gradually kille Unaccountably, in 20 % O_2 (air) the oxygen uptake at first increases considerab until, simultaneously with and at much the same rate as the 100 % O_2 curve

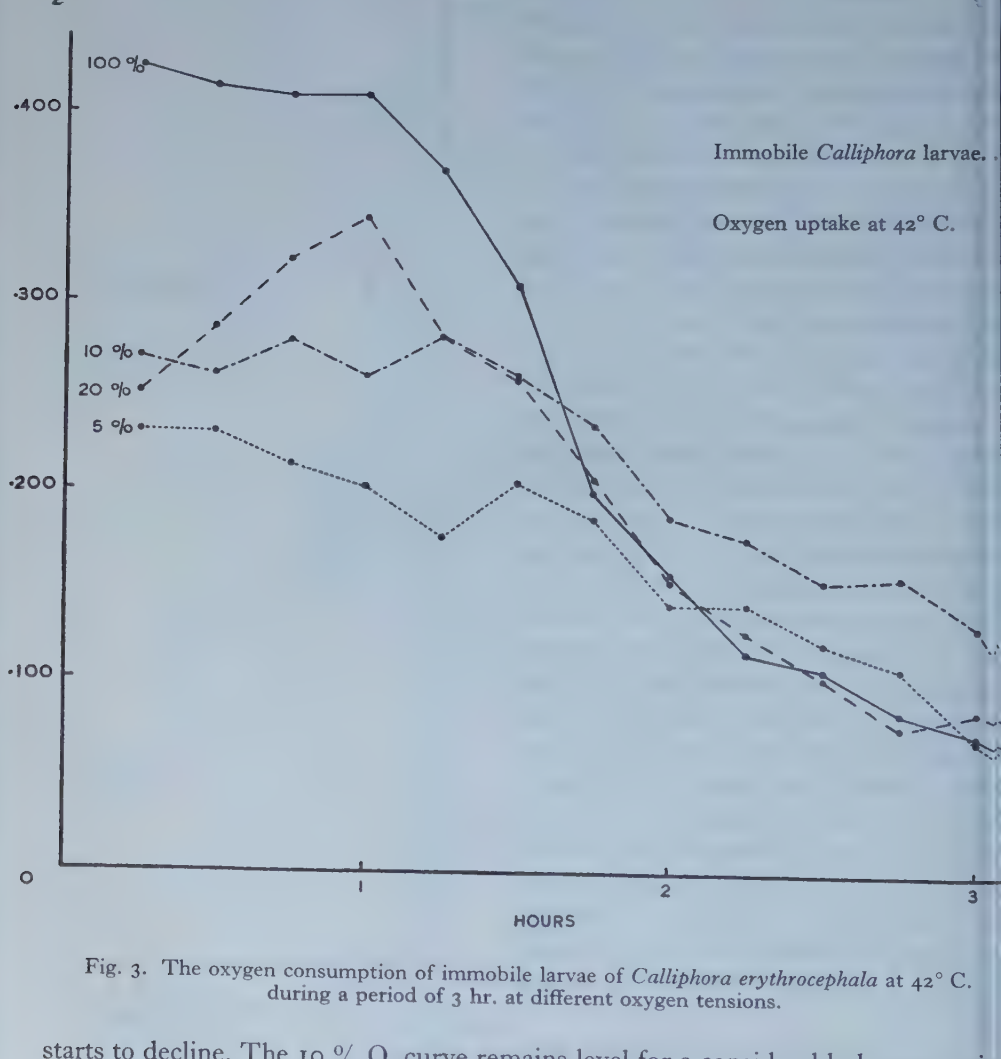

Fig. 3. The oxygen consumption of immobile larvae of *Calliphora erythrocephala* at 42° C. during a period of 3 hr. at different oxygen tensions.

starts to decline. The 10 % O_2 curve remains level for a considerably longer perio —1½ hr.—and then declines much more gently so that, as in Fig. 1, after 1½ h from the start of the experiment, it again lies considerably above the two oth curves. The 5 % curve, starting at a still lower level, soon begins to decline sharpl The curves of Fig. 4 show a similar picture. Here the oxygen consumption

o % oxygen from the onset is higher than at 50 and 100 %, whereas at 10 %, lthough starting at a considerably lower level, the O_2 uptake maintains its level or about 1 hr. 45 min., hereby crossing over the 50 and 100 % curves.

From these curves the conclusion must be drawn that high oxygen pressure, ar from being beneficial at high sublethal temperatures has actually a damaging ffect. Low oxygen pressure is also harmful, whereas a medium pressure seems to nable the organism best to resist injury to the respiration at high temperatures.

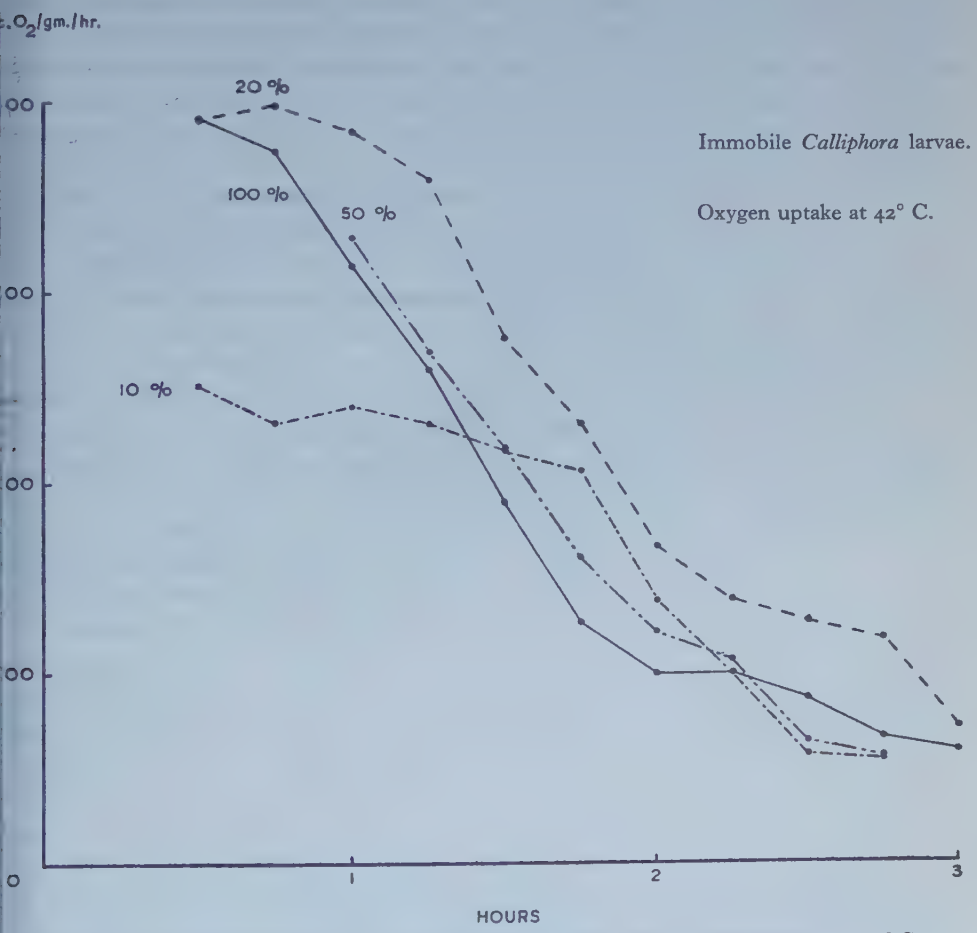

Fig. 4. The oxygen consumption of immobile larvae of *Calliphora erythrocephala* at 42° C. during a period of 3 hr. at different oxygen tensions.

From a consideration of Figs. 3 and 4 one is tempted to form the opinion that eat injury starts only from the moment when the curve, which expresses the basal netabolism, begins to decline sharply. This, however, is not so. If *Calliphora* arvae are exposed to 42° C., they become completely immobile (heat stupor) after pproximately 1 hr. From this stupor they seem to recover somewhat, but they nvariably die within 1–2 days. After exposure of 15 min. only, all larvae develop to

normal flies, but after 30 min. at 42° C., while still remaining active and mobil only 20 % pupate and develop into flies, the rest dying as larvae within 2 days. Aft exposure for 35 min. all die as larvae. Therefore the cellular respiration, as f as can be judged from the one figure of the oxygen uptake, remains unaffected f a considerable period after irreversible injury has been caused by the high tem perature.

So far the conclusion that high and low O_2 tensions are detrimental for th resistance to high temperatures is based only on the indirect evidence of the ra of O_2 consumption. This result has been confirmed with a direct method. Whe fly larvae are subjected to a given high temperature in different oxygen tension the larvae in pure oxygen and those at tensions of 2 % O_2 become immobilize more quickly than the larvae in air (Table I).

Table I. *Larvae of* Calliphora erythrocephala, *treated at* 41° C. *for* ½ *hr.*

	Immediately after treatment			One day later	
	Moving	Move only on touching	Motionless	Moving	Dead
100 % O_2	1 (just)	7	2	3 (just)	7
Air	3	5	2	5	5
2 % O_2	—	—	10	—	10

All died subsequently without pupating.

Table II. *Larvae of* Calliphora erythrocephala *treated at* 39° C. *for* 5½ *and* 6 *hr*

	5½ hr.			6 hr.		
	Pupae	Living larvae	Dead larvae	Pupae	Living larvae	Dead larvae
1 day afterwards						
80 % O_2	—	9	1	—	9	1
Air	—	10	—	—	10	—
7·7 % O_2	—	10	—	—	10	—
4 days afterwards						
80 % O_2	4	—	6	3	—	7
Air	9	—	1	7	—	3
7·5 % O_2	4	—	6	—	—	10

On choosing a lower temperature and a suitable period of exposure which doe not kill the larvae outright, and allows a certain percentage to pupate, it was foun that the ultimate number of pupae formed was greater in air than at either 80 % or 7·5 % oxygen (Table II).

DISCUSSION

The fact that increasing the partial pressure of oxygen does not protect a animal against the injurious effects of high temperatures has already been recorde by Winterstein (1905), who found that increasing the oxygen content of sea wate does not influence the inception of heat stupor in *Mysis* and in Medusae. He doe not, however, draw any conclusions from this observation.

Similarly Babak (1907), working with frogs, found that the resistance to lack f oxygen is not correlated with resistance to high temperatures. *Rana fusca*, hich tolerates oxygen deficiency better than *R. esculenta*, is paralysed within min. at 35° C., whereas the latter is paralysed within 3 min. at 40° C. Again, in dpoles of *R. esculenta*, the sensitivity to oxygen deficiency increases with age, hereas the sensitivity to high temperatures correspondingly decreases (Amerling, 908).

Mayer (1917) found that the resistance of corals to high temperature is independent of the oxygen tension at 6·6, 4·3 and 1·7 c.c. O_2/l. in sea water. On the ther hand, the sensitivity of different species to high temperatures was found to e similar to the sensitivity to CO_2. The author concludes that death at high mperatures may be due to the accumulation of acids, possibly H_2CO_3, but not asphyxiation by lack of oxygen. This is supported by the fact that corals die in a water saturated with CO_2 in less than 1 hr., but survive in the dark (to exclude xygen production by symbiotic algae) in sea water deprived of oxygen for 11 hr.

It is highly improbable that in our experiments death is caused by the accumulation of CO_2 in view of the efficient absorption of CO_2 by the $Ba(OH)_2$ in the vessel, nd of the well-established quick rate of diffusion of CO_2 in tissues. There may be, owever, an accumulation of lactic acid or other metabolites. It has been shown y Fletcher & Hopkins (1907) that the lactic acid content of isolated frog's muscles ncreases greatly when the muscles have been kept for 1 hr. at 40–50° C.

Hopf (1939, unpublished) investigated the influence of high temperatures on the ydrogen ion concentration of the blood of blowfly larvae. Choosing a temperature nd period of exposure which would kill the larvae eventually but not immediately, e established a lowering of the *p*H by 0·2 of the *p*H scale (e.g. 7·0 before treatment, ·8 after treatment).

Therefore all the evidence points to the conclusion that lack of oxygen cannot e the cause of death at high temperatures. Fig. 1 shows that at 42° C. the oxygen ptake is entirely dependent on the oxygen pressure outside the body. Previously Fraenkel & Herford, 1938) it was found that the oxygen consumption of ligatured lowfly larvae at 27° C. is almost independent of the oxygen pressure from 100 % own to about 8 %. According to Krogh (1916) and Gaarder (1918) one should xpect the oxygen uptake to be independent of the oxygen tension as long as there a positive oxygen tension in the tissues and "that the oxygen pressure becomes ne limiting factor only when the oxygen supply fails and the oxygen tension in ne tissues becomes zero". In agreement with our own results are those of Lindemann (1935) who found that the respiration of amphipods becomes independent of ne oxygen concentration at 10° C. above an oxygen content of 0·14 vol. %, whereas 26° C. the rate is wholly dependent upon the oxygen concentration. This would ggest that *Calliphora* larvae in air at 42° C. are actually suffering from the lack f oxygen. This, however, does not seem to be the primary cause of their death; r increasing of the oxygen tension, far from improving the situation, actually akes it worse. There can be no doubt that at 100 % O_2, the larvae are dying more uickly than at 20 and 10 %. Thus one is tempted to argue that the very factor of

high oxygen uptake might be detrimental for the animal, for lack of burnab material or insufficient removal of metabolites. One might expect, then, that ve low oxygen tensions, by preventing the organism from consuming too much oxyge might increase its resistance to high temperatures. Actually this has not been bor out. The optimum resistance has been found at 10–20 % oxygen tension. High as well as lower oxygen tensions proved injurious. This suggests that accumulati of metabolites which is equally favoured at high oxygen tensions because of the formation in vast amounts, and at low tensions, because of their insufficient oxid tion, might be the deciding factor, a view which is in agreement with the conclusio of Mayer reported above, and which is also supported by Hopf's *p*H determinatio

It has been shown that the oxygen consumption still appears to be normal a time when the damage caused by the high temperature is already irreparable, a for a considerable period afterwards. Winterstein (1905) has shown that the oxyg consumption of Medusae continues after they have already become paralysed l heat. Curves which show the dependency of the oxygen consumption on ten perature are usually constructed from individual determinations which are n extended over a sufficiently long period to ensure that the points near the maximu in the curve are representing the respiration of the normal healthy animal. Not on does the decline of the curve beyond the maximum demonstrate vividly the ons of heat coma but it is probable that in the temperature region at which maximu oxygen consumption is obtained and even at slightly lower temperatures, a simil falling off would have become manifest if the determinations had been extend over a sufficiently long period. One should therefore be very cautious in the i terpretation of the shape of such curves. This onset of irreversible damage possib accounts for some of the "breaks" in temperature-velocity curves which ha recently been an object of much discussion (Crozier, 1924–7; Belehradek, 193 Heilbrunn, 1937). If one were judging the resistance to high temperatures alo from such curves, one would reach the conclusion, that the increase of oxyg tension, which prolongs the curve further upwards (Fig. 1), would make t organism more resistant to high temperatures, whereas exactly the opposite the case.

SUMMARY

The oxygen consumption of blowfly larvae at sublethal and lethal hig temperatures at the beginning of the determination is entirely dependent on t oxygen pressure, but after about 1 hr. at 42° C. it is higher at 20 and 10 % parti pressure of oxygen than at either 100 or 5 %. Death at high temperatures is n due to lack of oxygen, but may be due to the accumulation of acid waste produc of the metabolism.

The basal oxygen consumption remains unchanged for some time after t organism has been irreversibly injured by the high temperature.

Blowfly larvae resist the damaging effect of high temperatures slightly bet in air (20 % oxygen) than in either very high (100 %) or very low (less than 10 % concentrations of oxygen.

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EXPERIMENTAL PRODUCTION OF FUNCTIONING REDUPLICATIONS—A TRIPLE AND A FUNCTIONING QUINTUPLE HINDLIMB IN THE FROG[1]

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(*Received* 26 *August* 1940)

(With One Plate and One Text-figure)

Experimentally produced functioning reduplications, triple and quintuple hind limbs of the frog have not yet been described.

Supernumerary limbs can be produced either by transplantation methods or by the methods described in this paper.

In order to prevent confusion between a reduplication of the primordium itself and a doubling after orthotopic transplantation of a limb bud, the author distinguished (1925) (i) reduplication of the limb primordium and (ii) pseudoreduplication consisting of an orthotopic transplanted limb of a donor and a regenerated limb of the host. This grouping was accepted by Smook (1934).

In the following description the above-mentioned terms will be applied in the sense given.

As this paper deals with supernumerary limbs of the primordium itself the present cases are more instructive for human and animal pathology than are the transplantation experiments. Reduplication of this kind reveals the biologic potency of the limb primordium as realized in abnormalities such as sometimes develop in man and animals.

Another problem dealt with in the present paper is a neurological one. The function of the triple and quintuple reduplications indicates some remarkable capacities of the central nervous system acting upon these multiple primordia since their earliest embryonic development.

There will be given in this paper a short preliminary record only of the function further details will be published later of the movements after they have been studied by cinematography.

MATERIAL AND METHODS

A total of 288 different operations were performed in the frog embryo from the neurula stage up to the 27 mm. tadpole stage. Two methods gave the best results:

(1) *Rotating method*: consisting of rotation of the hindlimb primordium.

[1] The work was aided by a Grant of the Medical Faculty Research Fund and the Sir Charles Hyde Fund of the University of Birmingham. The author wishes to tender his thanks to the Research Committee of the University for granting these funds.

(2) *Splitting method*: consisting of dividing the hindlimb primordium into two qual halves.

The rotating method can be applied to embryos over 7 mm. total length and tadpoles. The best stage for splitting the hindlimb bud is at the 20 mm. tadpole age when the hindlimb bud shows as a small round greyish swelling. More than 00 operations on neurulae at the tail bud and at later stages up to 7 mm. gave no esult.

For operations the author used small dishes partly filled with beeswax. The mbryo or larva was put under water into a depression in the wax cavity made of ich a size and shape as to fit its body snugly. The tadpoles were anaesthetized with hloroform water. By a specially constructed curved and ground iridectomy knife he hindlimb was circumcised with the aid of a binocular microscope. After lifting, he bud was rotated 180°, re-implanted and placed under a cover-slip for an hour, nd then the larva was transferred to a bowl of fresh water.

The splitting method was performed with the same iridectomy knife. The dpole was fixed by curved pins on a wax bed. The bud was halved from its dorso-nterior to its ventro-posterior end and a small piece of tail, cut from another larva as put between the two halves to prevent fusion. The larva was then transferred fresh water.

DESCRIPTION OF SUPERNUMERARY LIMBS

The following description will not include all the author's results which will e described in further papers.

Reduplications

ase 841. Splitting method.

The reduplication developed after splitting of the hindlimb primordium in the 2 mm. tadpole stage. The photograph (Pl. I, fig. 1) was taken 8 weeks after the peration. The total length of the frog from the tip of the snout to the tip of the il is 1·3 cm. The left thigh, the two thighs on the right side and the legs are 4 mm. ng. The feet of the three limbs are 6 mm. long, and both thighs of the reduplication re fused and are 3·3 mm. thick. The three legs are equal in calibre and are 1 mm. their thickest parts.

As has been shown in the forelimbs in amphibians and in the hand and the foot man (Brandt, 1925, 1931) the reduplications developing from a single primordium how mirror symmetry. The axis along which the arrangement of the limbs takes lace is a radial or tibial axis. There are very rare exceptions to this rule. The case the hindlimb of the frog confirms this and shows a mirror symmetry along its bial axis. The caudal member of the reduplication is a right hindlimb, the cranial he is a left hindlimb. Therefore the animal has two left limbs, a normal left on the ft side of the body and another reduplicated left hindlimb on the right side of the dy. The two reduplicated limbs function normally as if they were the normal mbs of the animal. As the animal possesses a left limb on the left side of the body hich functions synchronously with the left limb on the right side of the body,

the spinal cord is able to supply two left limbs, one of them on the right side of t body.

The movements of the reduplication consisted of flexion and extensic abduction and adduction at the knee and ankle during jumping. The fused thig of the reduplication were not movable at the hip joint. The left hindlimb of t reduplication moved synchronously and identically with the normal left hindlim the movements of the right hindlimb were co-ordinated with those of both l limbs.

The case represents the first observation of co-ordinated movement of expe mentally produced reduplicated hindlimbs in the frog.

These movements of the reduplication may be compared with those of a pseud reduplication in which, after transplantation of the right primordium of the fo limb of an embryo of triton taeniatus into the normal place of development of t right forelimb of another embryo, the author (1922, case 144) first observed 3 wee after the operation synchronous flexion, extension, abduction and adduction of t fully developed parts of the limb of the donor and of the normal regenerated rig limb of the host. The details of co-ordinated movements are described in Bran (1925, p. 224).

Case 1220. Birmingham, 1940. Operated 18 April (splitting method). Fix 10 June. Pl. I, fig. 2.

The reduplication is restricted to the foot showing the two members arrang in mirror symmetry along the tibial axis. There is a single thigh and a single l only. The right thigh is 24 mm. in length, the left one 46 mm. Contrasting wi the free movement in the former case the movements in this case were restricted slight flexion and extension of the ankle and slight co-ordinated movement of t fourth and fifth toes.

Triples

Case 1284. Birmingham, 1940. Operated 26 April (splitting method). Phot graphed 12 June. Pl. I, fig. 3.

The triple on the right side consists of a reduplicated foot and a round straig sprout joining the limb at its ankle. The reduplication shows mirror symmet along its tibial axis and consists of a right and left foot, according to the arrang ment of the toes. The shortest toe is the first, the longest is the fourth. The fif and the second toes are about the same length. There is one thigh and one l only and thus the triple is restricted to the foot.

The hip joint showed very slight movements of abduction and adduction. T third, fourth and fifth toes of the reduplication showed slight degrees of c ordinated flexion when the animal intended to jump. After sitting, the toes we extended to their former position. The sprout remained stiff throughout. Aft stimulating the sole the two reduplicated feet showed some irregular and unc ordinated movements, viz. isolated flexion or extension of single toes.

ase 1251. Birmingham, 1940. Operated 23 April (rotation method). Fixed 11 June.

The triple consists of a perfect reduplication of the same kind as described in se 841, and a third perfect limb developed at an angle of 90° to the reduplication l. I, fig. 4). The third limb is a normal right hindlimb on the right side of the dy. The reduplication lying ventral to it shows the typical arrangement of the rts in mirror symmetry. Whilst the ventral aspect of the hindlimb of the frog is pigmented, the two reduplicated hindlimbs show a conical area of pigment on is aspect. This pigmented area must therefore be considered the dorsal aspect of e reduplication which is fixed to the pelvis in a rotated position of 180°. The gment on the third hindlimb shows that it lies in the dorso-ventral position.

The movements of the third single hindlimb consisted of flexion, extension, duction and adduction at the hip, knee and ankle joints and were co-ordinated th those of the opposite side but restricted to a much lesser extent. Therefore is limb must be considered as a normal regenerated limb whilst the reduplication s developed from the rotated limb bud. The reduplication itself was fixed except r sluggish and limited indications of flexion and extension in the longest toes of th feet which appeared at a later time than the movements of the normal limb of e animal.

Quintuple

ase 1252. Birmingham, 1940. Operated 23 April (rotation method). Fixed 10 June.

The quintuple concerns the right hindlimb and shows a collection of limbs of fferent sizes and at different stages of development. At the time when the large mb (Text-fig. 1, no. 2) is fully developed, the foot (no. 4) is commencing to grow om the area of the knee of limb no. 3. It should be noted therefore that the uintuple does not represent a unity of limbs of equal age and that the limbs are ranged in very different positions. Seen from the ventral aspect (plate) their gnificance becomes clear. The ventral aspect of the thigh consists of a cranial pigmented half and a caudal pigmented half. The upper border of the pigented area indicates a line of demarcation between the originally rotated limb d and a new regenerated limb. Limbs 1 and 2 turning their dorsal pigmented pects ventrally must be considered as a unity which has developed from the tated limb bud. The upper part of the monstrosity consisting of limbs 3, 4 and 5 another unity and represents a regenerated stock. Limb 3 is a perfect regenerated ndlimb consisting of thigh, leg and foot in normal position, the accessory feet and 5 are rudiments growing from the area of the knee of the regenerated limb.

The anatomical feature of these limbs corresponds to the physiological. Slight ovements of flexion of the toes and of the ankle of limbs 2 and 3 were co-ordinated th identical movements of the normal hindlimb on the left side. The range of ovement in the normal left hindlimb was more extensive than in the quintuple. he hip joint and limbs 1, 4 and 5 were fixed.

Text-fig. 1. Case 1252. 1940. Rotation method. Photographed 46 days after operation. Quintu hindlimb. Limbs 1 and 2 belong to the originally rotated limb bud showing the dorsal pigmen side at the ventral aspect of the animal. Limb 3 is the normal regenerated right limb of the ani in normal position. Limbs 4 and 5 represent supernumerary feet arising at the knee of limb 3.

SUMMARY

1. The supernumerary limbs were produced by the rotating and splitti methods applied to the hindlimb bud in tadpoles of early stages of developmen

2. The two methods gave different results. Using the rotating method, rotated bud may develop a limb or multiples of a limb inverted at 180° and regenerated limb of normal position. Using the splitting method, two limbs mirror symmetry may develop besides a regenerated limb.

3. The function of supernumerary limbs is two-fold and may consist either co-ordinated and synchronous movements of fully developed limbs or parts limbs or the function may be restricted to some retarded and imperfect movemen

4. There is a great variety of form, structure, rate of development and arran ment of the parts of the multiples, to which a special function is related. C animal may show perfect co-ordinated movements of fully developed reduplicat limbs (case 841), another may show restricted co-ordinated movements (case 122 while others may show co-ordinated movements of parts of other supernumer limbs (cases 1251, 1284).

5. The co-ordinated movements in experimentally produced supernumera hindlimbs of the frog are to be regarded as an "integration of a series of reflexes and they are related to the same reflex mechanism which acts in the normal amb

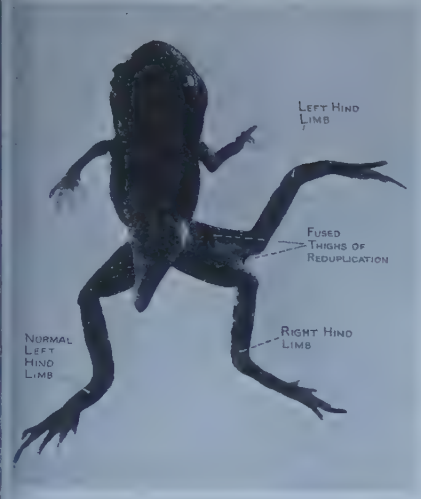

Fig. 1.

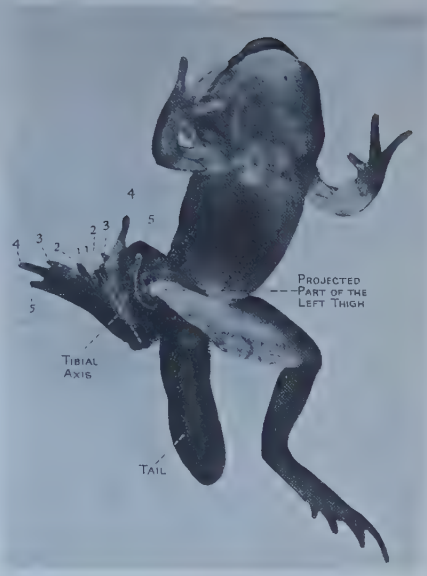

Fig. 2.

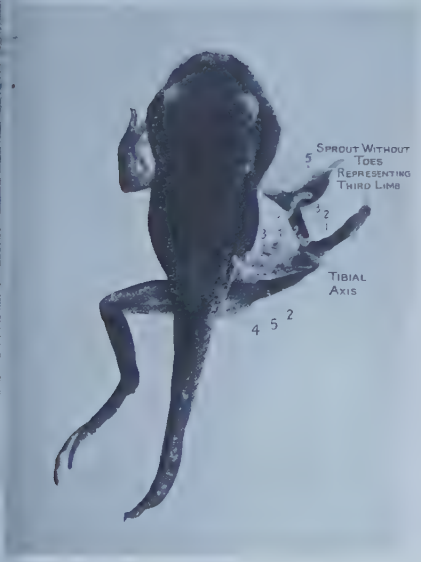

Fig. 3.

Fig. 4.

BRANDT—EXPERIMENTAL PRODUCTION OF FUNCTIONING REDUPLICATIONS—A TRIPLE AND A FUNCTIONING QUINTUPLE HINDLIMB IN THE FROG (pp. 396–401)

tory cycle of the toad (Gray & Lissmann, 1940). The retarded and imperfect novements in some supernumerary deformities indicate that disturbances of the ormal cycle are not the expression of a centrally determined rhythm.

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EXPLANATION OF PLATE I

ig. 1. Case 841. 1937. Splitting method. Photographed 2 months after operation. Fully developed eduplication showing mirror symmetry.

ig. 2. Case 1220. 1940. Splitting method. Photographed 53 days after operation. Reduplication f the foot. The toes are numbered to show their arrangement along the tibial axis.

ig. 3. Case 1284. 1940. Splitting method. Photographed 47 days after operation. The re-uplication of the foot is along the tibial axis. The sprout without toes represents a third limb.

ig. 4. Case 1251. 1940. Rotation method. Photographed 47 days after operation. Triple hind-mb. The fully developed reduplication of the hindlimb shows its dorsal pigmented side at the entral aspect of the animal. The third limb is partly covered by the reduplication.

THE OXYGEN CONSUMPTION OF FLIES DURING FLIGHT

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(*Received* 1 *September* 1940)

INTRODUCTION AND METHODS

In tables representing the rate of oxygen consumption of various animals, calcu lated per unit weight, by far the highest values are given by flying insects. Hithert the most reliable determinations have been made with honey bees and severa Lepidoptera, and values of 80–310 c.c. oxygen consumption per g. live weight pe hour have been recorded. One of the chief difficulties that has been encountere by previous workers on this question has been to obtain insects which will fl continuously in experimental conditions in a confined space. Most previou observers worked with freely flying insects which would not fulfil this condition i a satisfactory manner. To overcome this difficulty we worked with insects flyin in a fixed position, making use of the so-called flight reflex (Fraenkel, 1932). If a insect is suspended in mid-air in such a manner that there is no interference wit the free movements of the wings, flight starts as a reflex action when the support i withdrawn from the legs and lasts until the legs regain contact with a solid objec In actual practice, however, flight only lasts for a limited period, varying for dif ferent species and individuals, coming to an end through fatigue or other causes There are, nevertheless, several flies which may fly in the suspended condition fo periods of up to 1 hr. After trying out several species, including *Calliphora erythro cephala* Mg., *Phormia terranovae* R.D., and *Sarcophaga falculata* Pand., the mos satisfactory results were obtained with *Lucilia sericata* Mg., the sheep blow-fl which was exclusively used in the experiments now reported. When this fly i suspended in the following manner it will fly continuously for periods of 20 mi or even longer if it is in good condition. A small strip of paper to which is attache a piece of very thin wire bent into a hook is fixed to the dorsal surface of the thora of the lightly etherized fly with a patent quick-drying adhesive made from am acetate and cellulose in such a position that it does not interfere with the move ments of the wings. Flies thus prepared lived for the normal period after th treatment if fed on sugar. After each experiment the fly was killed, weighed aft removal of the suspension and dried to constancy of weight at 100° C. The wat content is usually about 70%, although figures as low as 60% and as high as 75% were occasionally found, these discrepancies most probably being due to differe states of nutrition.

The apparatus used in the first series of experiments was a modified Warburg espirometer, and in the second series a modified Barcroft respirometer. Both ieces of apparatus had a graduated side tube attached to them, similar to that lescribed by Dixon (1934, p. 8), so that readings could be made under constant ressure. The bottles had a capacity of about 60 c.c., and the fly was suspended rom the end of a tube connecting the interior with the outside air. The bottom f the bottles contained caustic potash as CO_2 absorbing agent.

The experiments were performed in a room kept at 27° C. by thermostatic ontrol in which the apparatus and the gas mixtures used in later experiments vere continuously kept so that only very short equilibration periods were necessary. Each insect suspended in the apparatus was allowed to fly as long as it would, and he oxygen uptake was measured at intervals timed with a stopwatch. These intervals vere intended to be 5 min., but as the flies would sometimes stop and start again he times of actual flight had to be measured accurately.

Table I. *Oxygen consumption of flies during flight in air*

Fly no.	Period of flight min. sec.	Oxygen consumption in c.c./hr./g. wet wt.	Av. oxygen consumption in c.c./hr./g. wet wt.	Av. oxygen consumption in c.c./hr./g. dry wt.	Wet wt. in mg.	Dry wt. in mg.
1	2 55	72·950	70·630	241·650	32·5	9·5
	5 0	68·310				
2	10 0	27·270	22·155	84·782	44·0	11·5
	5 0	20·450				
	5 0	20·450				
	5 0	20·450				
3	6 0	53·450	44·829	157·584	29·0	8·25
	3 0	48·275				
	3 0	44·830				
	2 0	41·380				
	2 0	36·210				
4	5 0	42·105	37·894	127·057	28·5	8·5
	5 0	37·890				
	5 0	35·790				
	5 0	35·790				
5	2 0	63·380	65·008	200·680	35·5	11·5
	3 5	87·705				
	5 0	43·940				
6	5 0	93·150	82·535	239·285	36·5	10·5
	4 0	71·920				
7	6 0	91·250	91·687	282·210	40·0	13·0
	5 0	87·000				
	5 0	93·000				
	4 0	95·500				
8	5 0	95·235	93·253	235·190	31·5	12·5
	3 6	95·212				
	5 0	89·312				
9	5 0	38·000	43·333	144·467	30·0	9·0
	5 0	44·000				
	5 0	48·000				

RESULTS

The consumption of oxygen was calculated both in terms of c.c. O_2 per g. dr weight per hour, and in c.c. O_2 per g. wet weight per hour. None of these figure for oxygen uptake were reduced to N.T.P. Calculations based on wet weight and o dry weight seemed to have no significant advantage over each other. The flies ma have drunk water or fed on sugar just before the experiment, while others woul secrete a drop of fluid from the mouth during the experiment or even defaecat All these factors influence the water content and necessarily account for much c the variation found in different experiments. Only those figures were included i the tables where one fly gave more than one reading, the similarity of the value of several readings being regarded as a check against possible experimental error

Table I represents the results of a series of experiments carried out with th modified Warburg apparatus (only one chamber, manometer open at other end It can be seen that the oxygen consumption of an individual fly remains fairl constant during subsequent periods. The variation of the values for different in dividuals are not considered to be unduly high in view of the unavoidable variation in dry weight and wet weight on which the calculations are based, and the individua differences in oxygen consumption which are well-known phenomena, particularl in mobile poikilothermic animals.

In the second series of experiments (Table II) a modified Barcroft-type apparatu was used, because it has a compensating vessel which corrects equilibrium effect

Table II. *Oxygen consumption of flies during flight in air, in pure oxygen, and in mixtures containing* 10 *and* 5 % *oxygen*

Fly no.	Gas	No. of determinations	Av. oxygen consumption in c.c./hr./g. wet wt.	Av. oxygen consumption in c.c./hr./g. dry wt.	Wet wt. in mg.	Dry wt. in mg.
1	Air	5	96·700	244·700	43	17
	Oxygen	5	96·670	247·060	43	17
2	Air	2	187·500	750·000	40	10
	Oxygen	2	180·000	720·000	40	10
3	Air	4	133·300	355·500	24	9
	Oxygen	4	138·300	368·800	24	9
4	Air	5	78·000	260·000	40	12
	Oxygen	5	86·000	286·660	40	12
5	Air	3	180·950	500·000	21	7·6
	Oxygen	4	214·200	592·100	21	7·6
6	Air	4	43·400	143·500	38	11·5
	Oxygen	4	68·400	226·080	38	11·5
7	Air	3	100·000	300·000	42	14
	10 % Oxygen	3	52·380	157·100	42	14
8	Air	3	180·000	450·000	20	8
	10 % Oxygen	3	55·000	137·500	20	8
9	Air	3	77·270	188·000	22	9
	10 % Oxygen	3	54·540	133·000	22	9
10	Air	3	172·400	550·500	29	9
	5 % Oxygen	3	48·270	155·500	29	9
11	Air	3	179·600	586·600	24·5	7·5
	5 % Oxygen	3	48·980	160·000	24·5	7·5

aused by temperature changes. In cases 1–6 the respiration was measured first in ir and then in pure oxygen. In three cases (1, 2, 3) the oxygen consumption was ractically the same in air and oxygen; in three other cases (4, 5, 6) it was only lightly higher in oxygen than in air. It therefore seems that an oxygen partial ressure of 21 % is sufficient to cover the considerable requirements during flight.

Finally, an attempt was made to determine whether and to what extent lowering f the oxygen tension would render the oxygen consumption during flight dependent n the oxygen tension. In an oxygen-nitrogen mixture, containing 10 % oxygen, he oxygen consumption becomes considerably less than in air (fly 7–9). In a nixture containing only 5 % oxygen only a few flies would fly. The oxygen con- umption is still less than in 10 % oxygen (fly 10, 11).

DISCUSSION

Table III contains a summary of the previous work done on the respiration of nsects during flight. As can be seen from column 2, the principal method applied vas to keep flying insects in a confined space and analyse the air for changes in xygen and/or CO_2 content. Parhon (1909) and Tauchert (1930) were not dealing

Table III. *Oxygen consumption of flying insects*

Insect	Method	State of activity	Oxygen consumption, g. wet wt./hr. (max. value)	Author
Hive-bee	Absorption and estimation of CO_2 output of a number of bees at once	Constantly changing, not continuous flight	34·000 c.c. No av. given	Parhon (1909)
Hive-bee	Gas analysis with single bees	"Running excited, sometimes flying"	73·000 c.c. No av. given	Tauchert (1930)
Hive-bee	Barcroft manometric method, with single bees	True flight	312·000 c.c. No av. given	Kosmin *et al.* (1932)
Hive-bee	Gas analysis, single bees	True flight	100·240 c.c. Av. 87·000 c.c.	Jongbloed & Wiersma (1934)
Moth, *Deilephila elpenor*	Interferometric gas analysis, single insects	True flight	Av. 6·690 c.c. CO_2	Kalmus (1929)
Butterfly, *Thais cassandra*	Gas analysis, single insects	Nicotine convulsion	216·000 c.c. No av. given	Raffy & Portier (1931)
Blow-fly *Lucilia sericata*	Warburg and Barcroft methods, single flies	True flight	187·500 c.c. (max. value found) Av. 95·580 c.c.	Davis & Fraenkel (1940)

vith the condition of continuous flight, and the work of Raffy & Portier (1931) was oncerned with butterflies injected with nicotine which did not fly but vibrated the vings convulsively. In the only other work in which a manometric method was ısed, the authors (Kosmin *et al.* 1932) give no indication of how the experiments

were arranged, and it is therefore impossible to discuss the results, which, compared with others, seem to give much too high values. Considering the differences of methods and material and the inherent difficulties of the experiment, the results of different authors seem remarkably similar. The average figures given for the bee by Jongbloed & Wiersma (1934) and for the fly by us are almost identical. The comparatively low values given for the hawk moth (Kalmus, 1929) can be explained by the much larger size and the smaller frequency of the wing beat of this moth compared with bees and flies. The rate of the wing beat of two similar Sphingids *Acherontia atropos* and *Macroglossa bombyliformis*, is 22 and 80 per sec. respectively; bees beat their wings at a frequency of 250 per sec. approximately (Magnan, 1934). We determined the rate of the wing beat of *Lucilia sericata* to be 160 per sec. approximately (measured acoustically with the aid of a microphone, an oscillograph and an oscillator).

It can therefore be stated that an insect of the size of a fly or a bee, which vibrates the wings rapidly, consumes oxygen at a rate of approximately 100 c.c. per g. live weight per hour. It is difficult to compare this value with that for the basal metabolism. This has never been measured in active adult insects like bees and flies. The basal metabolism of blow-fly larvae is about 0·5 c.c. per g. live weight per hour (Fraenkel & Herford, 1938), and the oxygen uptake of adult blow-flies which were *not* at rest, was determined as 2–3 c.c. per g. live weight per hour. It therefore seems that the ratio between the rest and the flight metabolism is in the region of 1 : 100. Flying insects maintain these extremely high rates for some time; a blow-fly may sometimes fly for more than 30 min. In man, during extreme muscular activity, the rate of metabolism increases by ten to fourteen times the normal resting exchange, but this cannot be maintained for more than a few minutes (Starling, 1936).

From our observations and those of other authors it seems reasonable to assume that the oxygen consumed during flight derives from carbohydrate metabolism. From the equation $C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$ it follows that $6 \times 22{\cdot}4$ l. of oxygen are required for 1 g.mol. carbohydrate, i.e. 180 g. Hence, a fly with an average wet weight of 31 mg. consuming oxygen at the rate of 95·58 c.c. per g. per hour would consume in 1 hr. 4 mg. of sugar. It was not found practicable to weigh flies before *and* after the experiment for determining the loss of weight during flight, because flies very often vomit or defaecate during the experiment and the periods of flight are often too short for accurate determination of loss of weight to be made. A series of determinations was, however, made to find out the amount of sugar which one fly may take up in a single meal. Flies were placed on a piece of cane sugar of known weight and the weight again determined after feeding. The results in four cases were 4·8, 5·1, 4·4 and 2·3 mg. It therefore seems that a well-fed fly carries sufficient sugar to allow flight for a period of 1 hr.

It was observed in some of the determinations that the oxygen consumption in subsequent readings fell off. It is difficult to say whether this was caused by depletion of carbohydrate stores in the flies, which were obviously in different experiments in a different state of feeding, or by gradual decrease in oxygen tension

n the small vessel. This falling off in the oxygen consumption was often very marked between the first and second reading. This phenomenon, which has often been noticed in the measurement of the respiration of cold-blooded animals, is due to the fact that animals are stimulated at first after being placed in a respiratory chamber and later "settle down". This effect in our experiments was certainly not a temperature equilibration phenomenon for the following reasons: rapid wing vibration warms the body of the fly and thus the surrounding air also; the air in the vessel would then expand and the reading would be *lower* instead of higher. After a time a new equilibrium would be reached and the effect would die away. Again, since the readings were regular and consistent on the whole, it seems unlikely that temperature changes influenced them. Also, the Warburg method seemed just as reliable as the Barcroft method in which there is a compensating vessel.

One may make the comment on flying insects in general that nearly all those which normally vibrate their wings rapidly like bees, wasps, flies, mosquitoes, Sphingids, and other Lepidoptera, feed on carbohydrates in the form of nectar. A notable exception to this rule are tse-tse flies which feed exclusively on blood. While the amount of blood sugar is certainly too low for sustaining enduring flight (approx. 0·1 g. %), it can be assumed that blood contains other food substances in an easily assimilable form. Humming birds, which are very insect-like in their habits and which vibrate their wings very rapidly and on account of their small size certainly have a very high metabolism, also feed mainly on carbohydrates.

SUMMARY

A method is described by which the oxygen uptake of the blow-fly, *Lucilia sericata* Mg., was measured during flight manometrically in a Warburg and in a Barcroft type of apparatus.

The average oxygen consumption in air for all the flies used was 95·580 c.c. per g. wet weight per hour. When flying in pure oxygen the rate of oxygen consumption showed no significant difference; in oxygen-nitrogen mixtures, containing 10 and 5 % oxygen, the rate was considerably less than in air.

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A FURTHER STUDY OF THE RELATION BETWEEN TOXICITY AND SOLUTION PRESSURE, WITH *POLYCELIS NIGRA* AS TEST ANIMAL

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(*Received* 5 *September* 1940)

(With Four Text-figures)

INTRODUCTION

Physiologists and biochemists have made many attempts to establish a relationshi between the physical and chemical properties of the metals and the degree o toxicity of their salts, and their views have varied from time to time to fit ne physico-chemical conceptions of the elements. Richet (1881, 1882) examined th relation between the toxicity of the metals and their atomic weight, and decide that no agreement was evident. Blake (1883), Botkin (1885) and Binet (1892) appea to have sought the solution of the problem in the periodic table of Mendelejeff, an Loeb (1902), mainly on the basis of his studies on the antagonistic action of ion seems to have regarded valency as the essential factor. Mathews (1904), whos hypothesis is the most satisfactory, showed that the physiological action of ion appears to be governed by their affinity for their electrical charges and that a re lationship therefore exists between the solution pressure of a metal and the degre of toxicity of its salts. Finally, Joseph & Meltzer (1909) discredited all these theorie and argued that the toxicity of metals bears an inverse relationship to the concentra tions in which they are normally present in ionic solution in the blood serum of th animal under experiment. Unfortunately, this study dealt with four metals only sodium, potassium, calcium and magnesium, and no attempt appears to have bee made to apply the theory to the more toxic elements.

Most of these earlier workers used frogs and other vertebrates as test animals Mathews, curiously enough, confined his experiments to the eggs of *Fundulus*, an the first review of the relation between the solution pressures of the metals and th degree of toxicity of their salts to a living animal is a study by the writer (Jones 1939), to which paper the reader is referred for a detailed exposition and discussio of Mathews's hypothesis. In this case the stickleback, *Gasterosteus aculeatus*, wa used as test animal, and a reasonably good agreement was observed.

The present paper presents the results of a similar investigation in which th test animal employed is one of the lower invertebrates, the Planarian *Polycelis nigr*

EXPERIMENTAL DETAILS

Various means have been devised whereby the degree of toxicity of dissolved ubstances to aquatic organisms may be assessed. Powers (1917) evolved an ad- irable, if somewhat complicated method which, unfortunately, is applicable only salts of the alkali and alkaline-earth metals, and Carpenter (1927) adopted a iterion applicable only to certain heavy metals. The only criterion capable of being pplied to all salts is that advocated by Reiner (1934); this is the threshold of xicity—the concentration at which toxic effect diminishes to zero and the rganism under experiment lives as long as the controls. This criterion was adopted the writer's study with *Gasterosteus*, the threshold of toxicity being taken as the ncentration at which the survival time of the fish attained 10 days, which is the pproximate average survival time of controls.

In tap water or glass-distilled water *Polycelis* will live for several weeks, though the absence of food or other stimulation it soon lapses into a state of complete rpidity. Accordingly, the determination of the exact threshold of toxicity for a lt demands lengthy experiment and presents certain practical difficulties; never- eless, a typical survival curve for *Polycelis* begins to rise very steeply when the lution is sufficient to permit a survival time of 48 hr., and comparatively slight rther dilution generally prolongs the survival time to many days. Thus for ractical purposes the concentration at which a survival time of 48 hr. is attained ay be taken as the threshold of toxicity, and in the present investigation this is the iterion adopted.

For each salt the determination of the critical concentration generally involved vo series of experiments. The first series, in which four or five widely spaced ncentrations were tested, served to indicate the required value within wide limits; ie second series, in which ten to fifteen solutions were used, covered a much arrower concentration range selected according to the results of the first series; is generally sufficed to indicate the threshold of toxicity with sufficient accuracy. hus, for example, in the case of nickel nitrate preliminary experiments with lutions containing 1000, 100, 10 and 1 mg. Ni/l. showed that a mean survival me of 48 hr. was attained somewhere between 100 and 10 mg./l. The second series which a closely graded set of concentrations covering this range was used showed at a 48 hr. survival time corresponded to 45 mg. Ni/l.

At each concentration 30 c.c. of solution was prepared and poured into a small etri dish. After a preliminary wash with glass-distilled water seven *Polycelis* were aced in each solution and the mean survival time of the animals was determined. was found most convenient to begin the experiment at about 3 p.m., as this per- itted more or less continuous observation over the 42–54 hr. interval.

All the solutions, stock and experimental, were made up with water distilled in ass, and the salts used were of A.R. quality when obtainable. After use the Petri shes were carefully cleaned before being used for another series of solutions, were nsed in tap water, washed with hot soap and water, soaked for some hours in water idified with nitric acid (to remove adsorbed ions), and finally rinsed and wiped

dry. Very dilute solutions, and solutions of salts which are unstable (e.g. MnC were renewed every 12 hr. The gold and silver series were kept in the dark, and on exposed to light when the animals were examined. The temperature at which t experiments were run could not be strictly controlled, and varied, with that of t laboratory, from 15 to 18° C.

RESULTS

The results are summarized in Table I. For each compound column 3 recor the concentration in mg. cation per litre at which a mean survival time of 48 hr. attained. The corresponding molar concentration (to two significant figures) is giv

Table I. *Thresholds of toxicity for* Polycelis nigra

Metal	Salt, etc.	Mg. cation per litre	Molar conc. cation	*p*H
Barium	$BaCl_2$, $Ba(NO_3)_2$	?	?	—
Sodium	NaCl	4370	0·19	6·0
	$NaNO_3$	1000	0·043	6·4
Strontium	$SrCl_2$	6600	0·075	6·6
	$Sr(NO_3)_2$	3500	0·04	6·6
Calcium	$CaCl_2$	2600	0·065	6·6
	$Ca(NO_3)_2$	1200	0·03	6·8
Magnesium	$MgCl_2$	970	0·04	6·2
	$Mg(NO_3)_2$	400	0·016	6·6
Manganese	*$MnCl_2$	700	0·013	6·0
	*$Mn(NO_3)_2$	660	0·012	6·0
Potassium	KCl, KNO_3	350	$9·0 \times 10^{-3}$	6·6
Aluminium	$Al(NO_3)_3$	110	$4·0 \times 10^{-3}$	4·2
Lead	$Pb(NO_3)_2$, *$Pb(CH_3COO)_2$	400	$1·9 \times 10^{-3}$	5·4
Chromium	$Cr_2(SO_4)_3$	75	$1·5 \times 10^{-3}$	3·6
Cobalt	$Co(NO_3)_2$	83	$1·4 \times 10^{-3}$	5·8
Nickel	$Ni(NO_3)_2$	45	$7·7 \times 10^{-4}$	6·6
Hydrogen	*HCl, *H_2SO_4	0·63	$6·3 \times 10^{-4}$	3·2
Arsenic	As_2O_3	40	$5·4 \times 10^{-4}$	6·4
Zinc	$ZnSO_4$	30	$4·6 \times 10^{-4}$	6·4
Cadmium	*$Cd(NO_3)_2$	2·7	$2·4 \times 10^{-5}$	6·6
Copper	*$Cu(NO_3)_2$, *$CuSO_4$	0·47	$6·3 \times 10^{-6}$	6·4
Gold	*$HAuCl_4$	0·6	$3·0 \times 10^{-6}$	6·0
Silver	*$AgNO_3$	0·15	$1·4 \times 10^{-6}$	6·6
Mercury	*$HgCl_2$	0·2	$1·0 \times 10^{-6}$	6·6
Iron	$FeCl_3$	>20·0	$>3·6 \times 10^{-4}$	3·2

* Solutions renewed every 12 hr.

in column 4. The *p*H of the solution at this concentration is also given, and it w be noted that some of the salts are appreciably hydrolysed. The hydrogen ion much less toxic to *Polycelis* than to *Gasterosteus*; this is clearly indicated by Fig. in which survival curves for both animals in dilute hydrochloric acid solutions a drawn. The survival time of *Polycelis* reaches 48 hr. at a *p*H of approximately 3· and HCl solutions of a *p*H of 3·6 or above have no definite lethal effect.

Solutions of ferric chloride have a toxicity approximately equal to that of H solutions of the same *p*H, and thus their toxic action appears to be due, mainly least, to the hydrogen ions set free by hydrolysis. The threshold of toxicity val of 20 mg./l. given for iron is thus a minimum value, and it is probable that *Polyce* would survive a greater concentration if the salt were not so extensively hydrolyse

The nitrates of sodium, magnesium, calcium and strontium are somewhat more oxic than the chlorides, apparently because the nitrate ion has a greater lethal ctivity than the chloride ion. Accordingly, the results with the chlorides represent nore accurately the degree of toxicity of these metals. Manganese nitrate and nanganese chloride are about equally effective, and in the case of metals of high oxicity the anion involved, provided it is ne of low toxicity, seems to play a minor art, the lethal action of the salt being reponderantly due to the cation.

In Fig. 2 the solution pressures of he ions are plotted against the threshold f toxicity values; the values for sodium, nagnesium, calcium and strontium are hose given by the chlorides, and the olution pressure values are taken from he sources cited in the previous study 1939). The mono-, bi- and tervalent ions ave been linked in separate series.

On comparing Fig. 2 with the corre-ponding figure for *Gasterosteus* (Jones, 939, p. 434) the general result is seen o be strikingly similar. The agreement or the tervalent metals is decidedly etter, aluminium being the least toxic f the series, not the most toxic. The ivalent elements, with the exception of ead, are arranged in the same order; ead occupies a curiously anomalous osition, and instead of being more toxic han cadmium is over 140 times less oxic. At high concentrations, however, ead is more rapidly fatal than copper Jones, 1937).

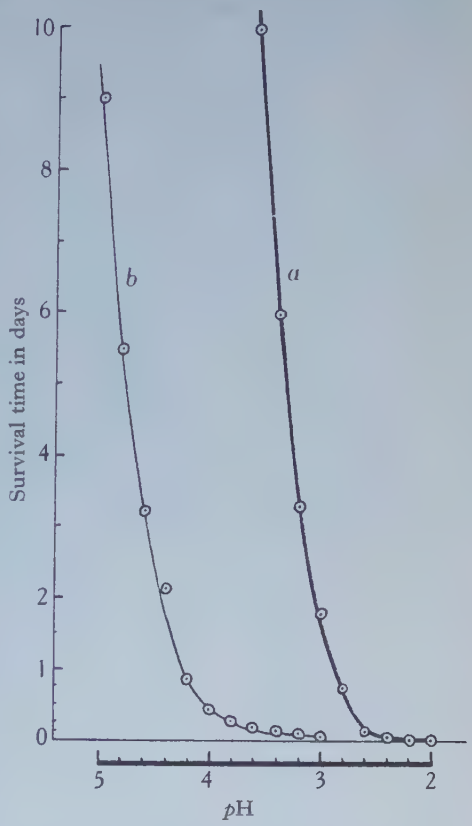


Fig. 1. *a*, survival curve for *Polycelis nigra* in solutions of hydrochloric acid. Each plotted point represents the mean survival time of seven animals. *b*, survival curve for *Gasterosteus aculeatus* (after Jones, 1939).

In Fig. 3 the thresholds of toxicity are iven in molar concentrations and the ons are linked up irrespective of valency. The general result is much the same, and hough a number of metals are irregular in position it is evident that the ions whose olution pressures have high negative values are comparatively innocuous, that hose whose values are positive are highly lethal, and that, very generally speaking, he more negative the solution pressure of an ion is the lower is its degree of toxicity.

It is somewhat doubtful whether arsenic is correctly placed on the solution ressure scale; the solution pressure value of +0·29 V. is given for the cation As''', whereas the active lethal agent in a solution of As_2O_3 is probably the anion of rsenious acid H_3AsO_3. According to its solution pressure gold should be more

lethal than silver and mercury, but it is possible that the toxicity of gold chlori solutions of low concentration is limited by their instability, for at great dilutio gold tends to abandon the ionic for the colloidal state.

It will be observed that no value is given for the threshold of toxicity of bariu Experiments with barium chloride and barium nitrate gave very irregular result thus, in a series of experiments with the nitrate, over the concentration range 300

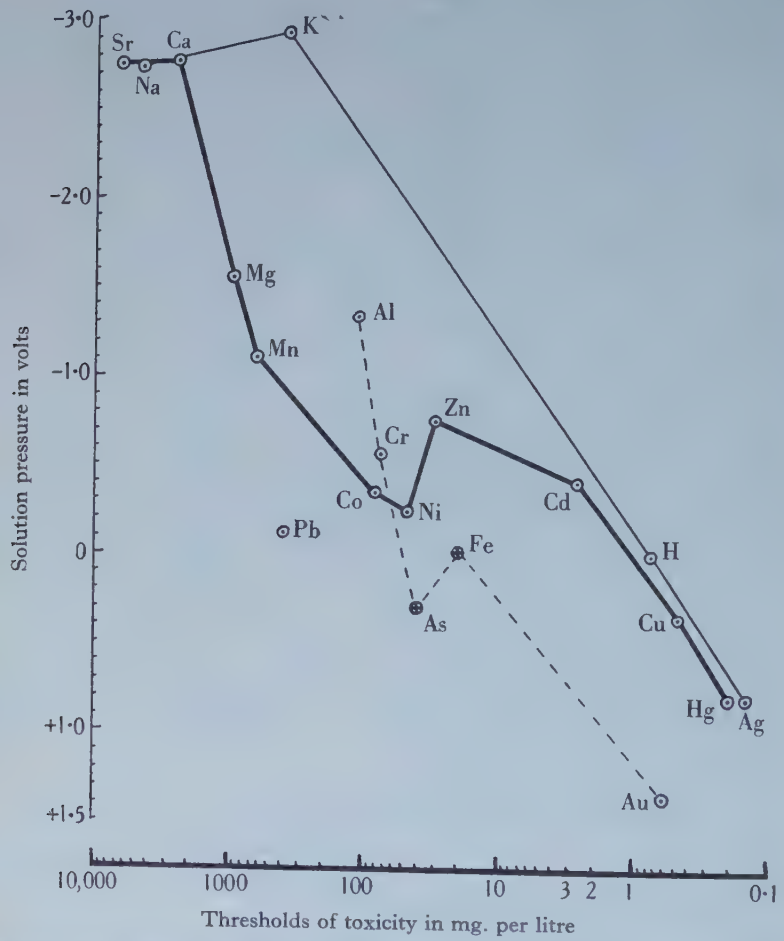
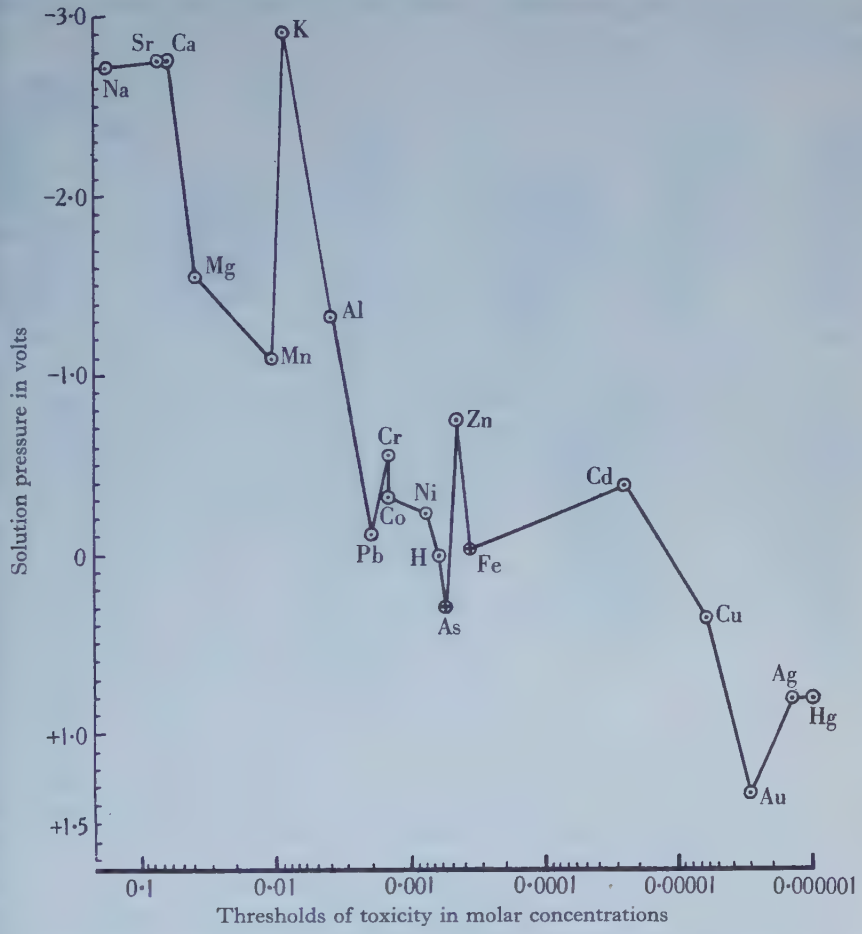


Fig. 2. The relation between the solution pressures of the ions and their thresholds of toxicity Explanation in text. Metals whose position on the solution pressure scale is uncertain, or whos threshold of toxicity cannot be definitely determined, are indicated by crossed circles.

60 mg. Ba/l., it was found that some animals died in all the solutions, some survive the 300 mg. solution for over 4 days, while some died in less than 24 hr. in th 60 mg. solution. Soluble salts of barium are said to impart violent and indis criminate stimulation to all kinds of involuntary muscle (Gunn, 1936, p. 146), an appear to be excessively stimulating to the musculature of *Polycelis*. A *Polycel*

mmersed in a $BaCl_2$ or $Ba(NO_3)_2$ solution soon begins to perform the most con-ulsive movements, the body is alternately expanded and contracted with great nergy, and in places the epidermis is so inflated by the pressure of the tissues within hat the epidermal pigment becomes sufficiently dispersed for these inflated areas o appear comparatively light coloured. Sooner or later the epidermis is so strained



ig. 3. The relation between the solution pressures of the ions and their thresholds of toxicity. xplanation in text. Metals whose position on the solution pressure scale is uncertain, or whose hreshold of toxicity cannot be definitely determined, are indicated by crossed circles.

hat it ruptures, and the tissues within begin to stream out. The animal continues o move until almost the whole of the body is in a state of disintegration; the ex-ruded protoplasm remains translucent, and does not appear to be coagulated by he electrolyte. Some impression of the reactions of *Polycelis* to a barium nitrate olution is given by Fig. 4.

In the case of the majority of the electrolytes studied the death of the animal is ccompanied by disintegration, which generally takes place after the cessation of

movement, but in some instances begins before the animal becomes inert. It i possible that certain salts other than those of barium owe their lethal effect, at leas in part, to excessive stimulation of the musculature of *Polycelis*. A complete reviev of the reactions of this animal to all the salts tested is beyond the scope of thi present investigation, but deserves further study. In particular the question of hov far the physiological effects of the less toxic salts is due to their osmotic pressur requires further examination.

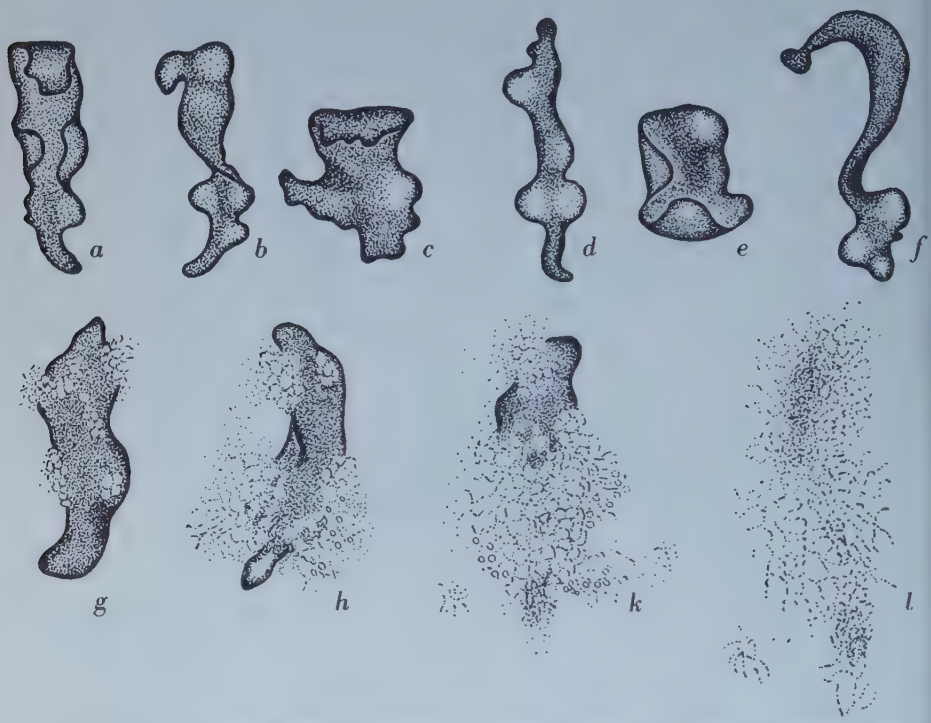


Fig. 4. Behaviour of *Polycelis nigra* in solutions of barium nitrate. *a–f*, typical attitudes assume during the earlier part of the survival time; *g*, *h*, *k*, rupture of the body and extrusion of the tissues *l*, final complete disintegration.

In the earlier investigation with *Gasterosteus* it was pointed out that the majorit of the salts studied bring about the death of the fish as a result of reactions whic take place outside the body, and that their rapidity of effect is not influenced by th speed with which they penetrate living tissues. It was therefore concluded that thi factor was largely responsible for the good measure of agreement observed betwee solution pressure and toxicity. In the case of *Polycelis* the lethal action of all the ion tested probably involves their penetration of the tissues of the animal, and it i somewhat interesting to find that when matters are complicated by the entry of th permeability factor a reasonably good agreement is still displayed.

SUMMARY

1. The degree of toxicity of the hydrogen ion and the ions of eighteen metals to *Polycelis nigra* (Müller) has been assessed by determining in each case the concentration the animal survives for approximately 48 hr. at 15–18° C.

2. On a mg./l. basis their order of increasing toxicity is:

Sr Na Ca Mg Mn'' Pb'' K Al Co'' Cr''' Ni'' As''' Zn Cd'' H Au''' Cu'' Hg'' Ag

3. On a molar concentration basis the order is as follows:

Na Sr Ca Mg Mn'' K Al Pb'' Cr''' Co'' Ni'' H As''' Zn Cd'' Cu'' Au''' Ag Hg''

4. The position of iron is uncertain; the toxicity of ferric chloride solutions appears to be due to their acidity. Irregular results were obtained with solutions of barium salts, which appear to effect excessive stimulation of the musculature of the animal and induce convulsive movements which eventually result in the rupture of the body and extrusion of the tissues.

5. On a mg./l. or molar concentration basis there is a decided relationship between the solution pressures of the metals and the degree of toxicity of their salts, the general result being very similar to that obtained in an earlier investigation with *Gasterosteus aculeatus* as test animal. This relationship suggests that the degree of toxicity of ions is largely determined by their affinity for their electrical charges, this affinity determining the readiness with which they tend to abandon the ionic state to enter into chemical combination with protoplasmic compounds.

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SECOND-ORDER OLFACTORY AND VISUAL LEARNING IN THE OPTIC TECTUM OF THE GOLDFISH

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(*Received* 10 *September* 1940)

(With Eight Text-figures)

INTRODUCTION

Many investigations of the effect of removal of portions of the brain on the powers of learning have been made by observation of the changes in the reaction given to a particular stimulus during a period of training in which the stimulus is associated with food as "reward" (see Lashley, 1937; Jacobsen, 1939). More complex learned reactions, such as conditioned reflexes of the second order (see Pavlov, 1927), have been little used in studies of this kind. In such second-order experiments, instead of food being given as "reward" during training, a second sensory stimulus of a different character is given, which has been associated with food during a preliminary training period. Removal of the sensory areas concerned from the brain of an animal trained in this way, should throw light on the type of mechanism whereby second-order learning is set up. For this purpose the most suitable condition is one in which the sensory centres are sufficiently widely separated for lesions to be made interrupting the connexion between them.

The brain of the Teleostei provides an opportunity for an experimental investigation of this type, since the areas receiving primary olfactory and optic fibres are widely separated. Also there are no projections forward from the midbrain and dorsal thalamus into the telencephalon (see Kappers *et al.* 1936), and interaction of olfactory and optic impulses cannot therefore take place any further forward than in the midbrain roof.

The experiments described in this paper were designed, firstly, to discover whether second-order olfactory learning with optic stimulation as "reward" is possible in the goldfish, and, secondly, whether injury to the optic tectum interferes in any way with this learned response.

PREVIOUS WORK ON LEARNING IN TELEOSTEI

Mobius (1873), confirmed by Triplett (1901), was the first to record learning in Teleostei. When a pike was kept separated from a number of minnows by a glass partition, it ceased snapping at them through the glass after a while, and when the glass was finally removed, never attacked them.

Further instances of learning in fishes have been reported by a great many workers. Thorndike (1899), Churchill (1916), Szymanski (1918) record maze learning, Russell (1931) the learning, by six specimens of *Gasterosteus*, of a detour into a glass pot to obtain food, and Spooner (1936) a fuller investigation of detour learning in the case of the wrasse *Crenilabrus*, giving numerical data in the form of learning curves. Bull (1928–37), in a series of papers, gives a great mass of data on conditioned responses to food and shock in a variety of teleostean species. Confirming the results of Froloff (1925, 1928), he was able to establish conditioned responses in *Labrus*, *Crenilabrus*, and *Blennius* to visual stimuli; by differential training the fish could be taught to discriminate between monochromatic green, yellow, and violet, but not intensity differences. In *Crenilabrus*, *Anguilla*, and a number of teleostean species, conditioned responses to vibratory stimuli were possible, as were responses to gustatory stimuli and to changes in temperature and salinity. From such data Bull was able to obtain knowledge of the powers of discrimination shown by certain teleosts in these modalities, but failed to establish a response in *Blennius* to an artificial olfactory stimulus.

A number of other workers have also used the learning method in the investigation of the sensory capacities of the Teleostei: Trudel (1929) and Klenk (1930)—taste; Herter (1929, 1930)—optic training to size, brightness, form, and colour differences—tactile training to surface texture.

Schiller (1933) claims to have shown "intersensorielle transposition" in *Phoxinus*. The animals, first trained to select the lighter of a simultaneously presented pair of lights, were then given a pair of smells (a "lighter" and a "darker" in the sense of Hornbostels, 1931), and chose the "lighter".

Nolte (1932) studied the localization of learned associations in the fish brain. In partially or totally decerebrated *Phoxinus* and *Gasterosteus* he found no gross motor disturbances. When these fish were trained to give a feeding response to the presentation of a certain colour, decerebration or removal of the habenular ganglion failed either to abolish the learning, or to impair visual form discrimination. Nolte concluded that in these species the forebrain is mostly concerned in olfaction. Hosch (1936) also found in *Phoxinus* and *Gobio* that learned visual form discriminations are unaffected by total forebrain removal, but Noble (1936, 1937) and Wielbalck (1937) have found forebrain lesions to affect mating and schooling behaviour in certain species of Teleostei. In fact, as Meader (1939) suggests, there is probably as much variation in the forebrain physiology of Teleostei as there is in forebrain anatomy. For example, in some species olfaction may be paramount while the eyes are poor; in schooling species the forebrain may effect the coordination necessary for this type of behaviour; in species with complex breeding habits and a predominantly "visual" mode of life the forebrain may be concerned in breeding behaviour, and the large hypothalamo-telencephalic tract conduct forward the necessary impulses from visceral centres.

Sears (1934) studied the effect of lesions of the optic tectum upon purely visual conditioned reflexes in the goldfish. A jet of water was used as the unconditioned stimulus and light as the conditioned. An associated response was set up within

fifty trials, and removal of large areas of the tectum did not abolish it or destroy the capacity for relearning. Large portions of the tectum were left intact laterally, however, and the possibility that this form of learning is mediated by these remaining portions is not excluded.

MATERIAL AND OPERATIVE TECHNIQUE

Eleven specimens of the goldfish (*Carassius auratus*) were used in this work. kept throughout the experiments in glass aquaria provided with running water from the laboratory supply, and transferred daily to the experimental room and back again. The temperatures of the aquaria and the experimental tank were taken before each day's set of trials, and approximately equated by the addition of either hot or cold water to the latter. During an experiment the animals were not fed except when in the experimental tank. The food used was ant pupae, since this was easy to standardize in quantity.

Operations were performed under 2% urethane anaesthesia on animals taken out of the water and wrapped in a wet cloth. Incisions were made into the dorsal aspect of the head with a small scalpel, and a longitudinal rectangle of skin and skull either turned back or removed altogether. Cuts were made in the optic tectum with either a very small pair of scissors, or a cornea knife. The brain is very easy of access. There was no excessive bleeding, and recovery was rapid. The skull flap was not always replaced, as the wound became stopped by a plug of coagulated blood, but in spite of the absence of aseptic precautions the fish all survived until killed for histological examination. Each fish was killed by decapitation, and the whole head fixed in a solution of 4 parts of formaldehyde in 96 parts of water. After some days in the fixative the brains were removed and prepared for sectioning and staining by the modified Weigert technique described by Sheldon (1912). The sections were used to determine accurately the extent of the lesions made (see Fig. 7 below). In order to map the lesions, the total number of sections (T.S.) occupied by the optic lobes was counted and divided by nine. The extent of the lesion in sections corresponding to each of eight equidistant points through the lobes was then measured, and plotted on to standard outlines of the midbrain. Three views of the midbrain in outline were used: a dorsal, a left lateral, and a right lateral. These were obtained by reconstruction from suitable series of normal brains.

THE ABSENCE OF MOTOR DEFECTS AFTER FOREBRAIN AND TECTAL LESIONS

A number of workers have investigated the effect of forebrain and midbrain lesions in Teleosts upon the movements of swimming and equilibrium (see Ten Cate, 1935).

In the case of the forebrain, lesions are without effect upon swimming or equilibrium (see also Meader, 1939). Observation of the movements of two fore-brainless fish during the present work confirmed this conclusion.

Lesions of the base of the midbrain (interpeduncular region) cause forced movements (Ten Cate, 1935). Reisinger (1926) found profound disturbances of movement and equilibrium after lesions of the valvula cerebelli. This result is confirmed by Tuge (1934). Rizzo (1932) concluded that lesions of the midbrain as well as of the cerebellum have an effect upon muscle tonus, locomotion, and equilibrium, but Ten Cate (1935) criticizes his results on the grounds that his operative methods did not allow of a sharply localized lesion. In fact, there is reason to believe that superficial midbrain lesions in the Teleost, where only the tectum and not the underlying valvula cerebelli is involved, produce little disturbance of locomotion or equilibrium. In the present work, unilateral removal of the tectum without damage to the valvula was attained in two animals: they swam and fed normally. In two further animals, where more or less severe damage to the valvula had occurred, circling movements similar to those recorded by Tuge (1934) occurred. In all the four animals used for the critical experiment, where the tectum had been variously damaged without damage to the valvula, no disturbances were observed.

METHOD OF TRAINING

Fish were trained in a tank divided into three compartments. The final reaction in each trial was passage from one compartment into the next through a hole in the partition separating them, and in no case was a trial ended until this reaction had been made. In the first-order learning situation the fish was placed in a compartment and the optic stimulus presented until the fish passed into the next compartment, where it was given food as "reward". After a period of training in this way with light and food the fish was trained in the second-order situation. It was placed in a third compartment and the olfactory stimulus presented. It eventually passed through the partition hole into the next compartment. This compartment was the one in which the optic stimulus was presented in the previous training, and this stimulus was given therefore instead of food as "reward".

At the beginning of every experiment the fish passed through the hole at random, but, as learning proceeded, passage through the hole became more frequently a result of the presentation of the stimulus. As an index of the course of learning the time elapsing between the presentation of the stimulus and the passage of the fish through the partition hole (reaction time) was taken for every trial. As the fish learnt, this became on the average shorter for each successive day's group of trials. Disturbances of learning appeared as considerable increases in average reaction time.

APPARATUS

This consisted of a galvanized iron tank $35 \times 23 \times 18$ in., divided primarily into a larger and a smaller compartment by a partition 12 in. from one end (Fig. 1, *A* and *B*). In the midline of this partition, 6 in. from the floor of the tank, was a hole 3 in. in diameter (Fig. 1, *D*). The tank was kept filled with water to a depth of 9 in. In compartment *B* a 60-watt electric light bulb (Fig. 1, *G*) was fixed by means of a wire frame at the level of the rim of the tank, exactly in the centre of the com-

partment. In compartment *A* a similar bulb, enclosed in a 3 in. diameter tube the mouth of which was covered by a disk of stretched white paper (Fig. 1, *F*) hung on the partition vertically above the hole *D*. The paper disk at the end of the tube was exactly 1 in. above the water level. The whole of the inside of the tank was painted a dull black.

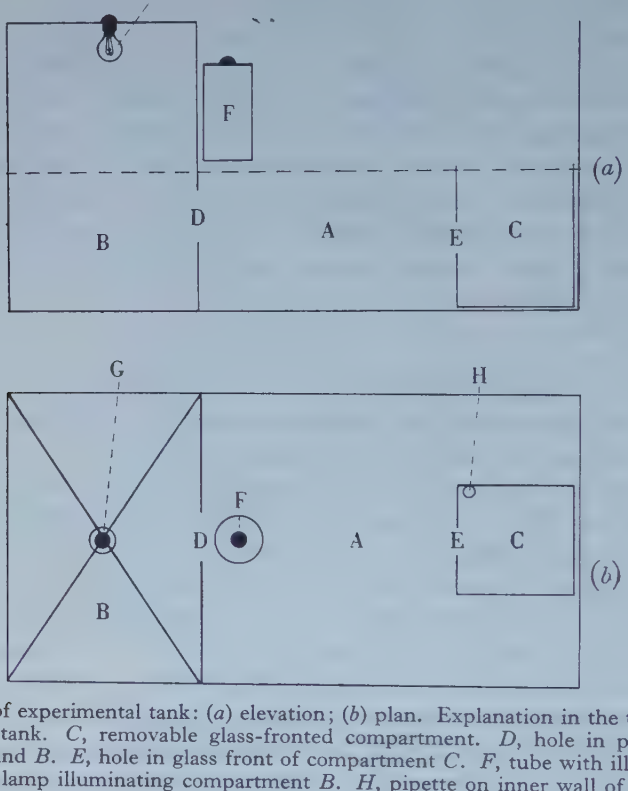

Fig. 1. Diagram of experimental tank: (*a*) elevation; (*b*) plan. Explanation in the text. *A*, *B*, main compartments of tank. *C*, removable glass-fronted compartment. *D*, hole in partition between compartments *A* and *B*. *E*, hole in glass front of compartment *C*. *F*, tube with illuminated disk at its lower end. *G*, lamp illuminating compartment *B*. *H*, pipette on inner wall of compartment *C*. Dotted line shows water level in tank.

The third compartment (Fig. 1, *C*), used in the experiments involving olfactory stimulation, consisted of a black iron box $8\frac{1}{2} \times 9 \times 9\frac{1}{2}$ in., open at the top, and one of whose sides consisted of a glass plate, in the exact centre of which was a hole 2 in. in diameter (Fig. 1, *E*), the thickness of the glass in the hole being painted black. Compartment *C* stood in compartment *A*, the glass front facing the partition between compartments *A* and *B*, and the hole in the glass exactly on a line with the hole *D* in this partition. The back of compartment *C* rested against the end wall of compartment *A*. Attached to the side wall of compartment *C* was a small pipette, used to introduce the odorous substance when olfactory stimuli were in use.

The experiments were conducted in a small dark room in the basement of the laboratory, and vibrations were reduced as far as possible by resting the tank on a number of rubber pads. The only illumination in the room during the course of a

run, apart from the lamps in the tank, was that provided by a faint red bulb. The behaviour of the fish during a trial was watched from as far away as was compatible with seeing the fish pass through hole *D*, great care being taken not to make unnecessary movements during the trial.

Prior to each day's trials, each fish was taken from the aquarium, placed in a large white dish in the experimental room, and left to become dark-adapted.

Hafen (1935) has shown in *Phoxinus* that rate of learning does not depend upon the number of trials given per day, but only on the absolute number of trials given. Thus no effort was made to keep rigidly to a definite number of trials per day. A maximum of six and a minimum of four was given. The fish seemed disinclined to feed after six trials.

RECORDING

The results are given in the form of learning curves. In these curves the average reaction time[1] of each fish for each successive day of the experiment is plotted against the duration of the experiment in days. When these curves are compared

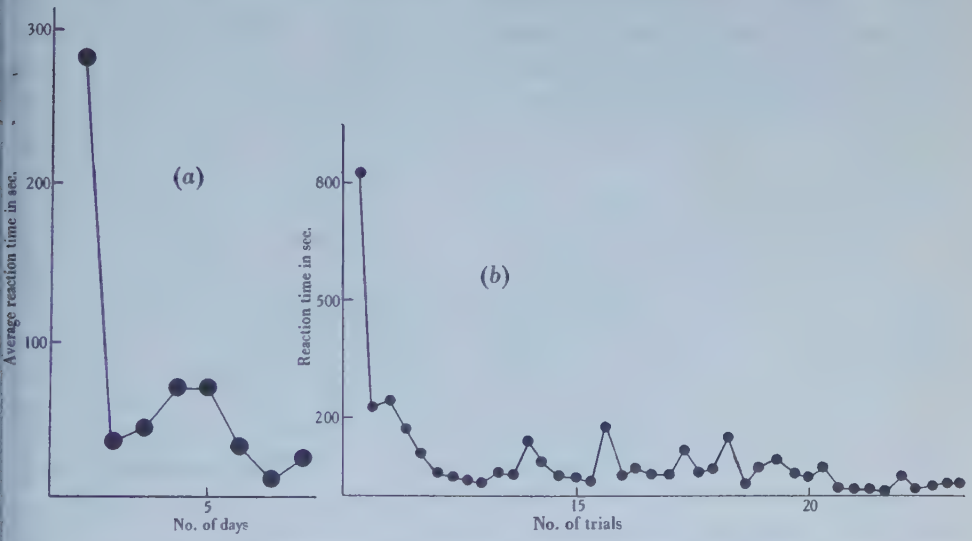

Fig. 2. Comparison of curves obtained by plotting: (*a*) average reaction time per day against number of days of experiment, and (*b*) reaction time per trial against number of trials.

with those obtained by plotting individual reaction times against number of trials (see Fig. 2), it is found that the day-average curves still preserve the general shape and more striking features of the plot of individual trials, while the irregularities of trial-to-trial variation are smoothed out.

[1] This average was obtained by dividing the sum of the reaction times of the individual trials of any one fish on any one day by the number of trials given on that day.

LEARNING IN RESPONSE TO A VISUAL STIMULUS

The experiments described in this section were made in order to discover whether it was possible for the goldfish to learn to associate food in compartment *B* of the experimental tank with the visual stimulus provided by the illuminated disk in compartment *A*.

Experimental procedure. Compartment *C* (see Fig. 1) was not used in this series of experiments and was not placed in the tank. In every trial the light in compartment *B* was first switched on, and the fish transferred from the white disk in the experimental room direct to compartment *A* and left for 10 min. During this time it rarely passed through the hole. Then the disk was illuminated by switching on the lamp in tube *F*, and the stop-clock started. As soon as the fish passed into compartment *B*, four ant pupae were supplied to it and the reaction time recorded. At the end of each trial the fish was removed from the tank and replaced in the dish, being captured in either compartment at random, so that it should not associate capture with any particular region of the tank. Any excess food was then removed from the tank, and further trials given at intervals of half an hour.

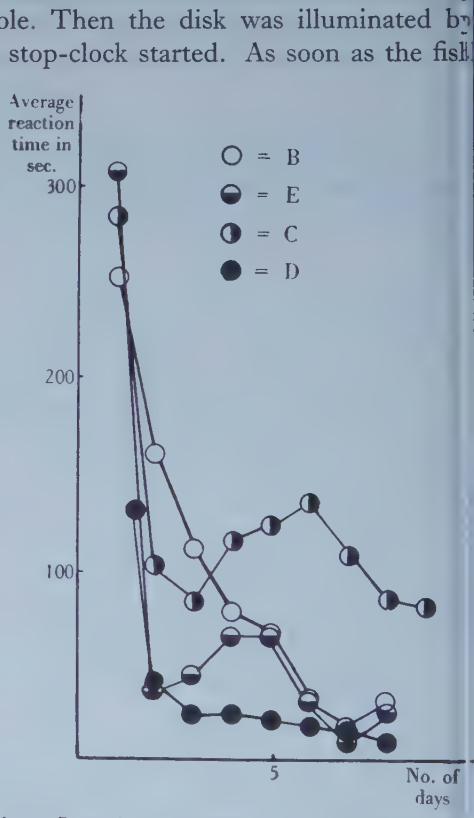

Fig. 3. Learning in response to a visual stimulus. Day-average curves for animals B, C, D, E.

Results. Four fishes were used. The results are shown in Fig. 3. These learning curves show an initial marked decrease in average reaction time, which subsequently varies between lower and narrower limits. The initial decrease shows that after a number of presentations of the illuminated disk in compartment *A* in association with food in compartment *B*, the fishes pass more quickly into compartment *B* upon presentation of the illuminated disk than they did at the beginning of the experiment.

Individual animals show great differences in the form of the curve after the first decrease. So many unknown factors may be involved here, that it seems unprofitable to consider individual variations in this region of the curve.

In the period of initial decrease all four curves have approximately the same slope, although they flatten out at different levels. The initial rate of learning is thus approximately the same for these four animals. After that it is subject to great individual variation.

These experiments were made as preliminaries to those described below, solely to ascertain whether visual learning of this type was possible in the goldfish, and so no tests of retention, or of the form of the curve of experimental extinction, were made.

LEARNING IN RESPONSE TO A COMBINATION OF VISUAL AND OLFACTORY STIMULI

A more complex association was then set up between food and combined optic and olfactory stimuli.

Strieck (1925) has shown in *Phoxinus* that gustatory response can be set up only to the four primary taste qualities apparent to man. Operative removal of the forebrain did not upset a discrimination learned between any one of these tastes and any other, but in the case of a discrimination learned between any one of the substances scatol, cumarin, artificial musk, and any other, this operation destroyed the discrimination. In intact animals this second discrimination could be made at much more dilute concentrations than the former. Strieck concludes that *Phoxinus* tastes and smells the same classes of substances as man.

In the present work amyl acetate was used as an olfactory stimulus. It was found that lesions anterior to the known mesencephalo-bulbar connexions abolished the learned reaction (the gustatory centres are situated in the medulla oblongata), and, since a small concentration was used, it seems safe to conclude that the effective stimulus was olfactory rather than gustatory.

Experimental procedure. Compartment *C* was necessary for this series of experiments, and the experimental procedure described in the preceding section consequently modified.

The illuminated disk in compartment *A* provided the visual stimulus. To serve as olfactory stimulus a constant small volume of amyl acetate ($\frac{1}{2}$ c.c.) was introduced into compartment *C* at every "smell" trial through the small pipette fastened to its wall. In order to control the possibility that this very small stream of fluid might act as a stimulus in itself, the pipette was used in all trials; in those in which an olfactory stimulus was not required, amyl acetate was replaced by water.

Where an olfactory stimulus is concerned the animal must be swimming in uncontaminated water prior to the introduction of the stimulus at every trial. Compartment *C* was used to avoid the labour of emptying the whole tank at the end of each trial. At the end of a trial compartment *C* alone was emptied of contaminated water. Then a vaselined glass plate was placed over the hole *E* and the compartment refilled with clean water and replaced in the tank. Immediately before the stimuli were presented this glass plate was removed by a waxed thread to which it was attached, and diffusion into compartment *C* from the main tank in the short time between this and the release of the stimuli was regarded as negligible. To avoid this glass plate being included as part of the stimulus complex in "smell" trials, it was made a constant feature of all trials. The main tank was cleaned out at the end of each day.

Thus the procedure for these experiments was as follows:

(1) Filling and placing compartment *C* in compartment *A*.

(2) Introduction of the fish into compartment *C* and leaving for 10 min.

(3) Removal of the glass plate.

(4) Switching on the illumination of the disk, starting the clock, and releasing fluid from the pipette in compartment *C*.

(5) Supply of four ant pupae to the fish in compartment *B* at the completion of the reaction, and recording the reaction time.

Results. Two fish only were used. The results are shown in Fig. 4. Two measures of the reaction were taken: first, the time taken by the fish to pass from compartment *C* into compartment *A* (*first response*), and secondly, the total time from the beginning of the trial to the passage of the fish from compartment *A* into compartment *B* (*second response*). These two responses are plotted as day averages on the same graph in Fig. 4. In this and all subsequent experiments, after the initial drop in the learning curve had been recorded, and disturbances of learning were being investigated, trials were not proceeded with after 11 min. had elapsed without reaction. These trials are plotted on the curves as points on an "eleven minute plateau".

The fishes were first trained to make both first and second responses to the visual stimulus of the illuminated disk. When a good response was established, they were presented for 3 days with amyl acetate in compartment *C* as an additional stimulus. Then a return was made to the visual stimulus alone for a further 2 days. In both fishes this removal of the olfactory stimulus gave a rise in the curves denoting disturbance of the learning. The olfactory stimulus had thus come to play some part in the complex of stimuli necessary to call forth the learned response.

Next the visual stimulus was omitted during the trials of 2 days, and another rise in the curve was obtained. Then the fishes were given 3 days of trials in which the full complex of optic and olfactory stimuli was given together with food to reinforce the learning. Finally, the full complex of stimuli was presented for a further 3 days, only no food was given. These trials were accompanied by ascent of the curve to the "eleven minute plateau". The ascending curve of experimental extinction so obtained has a more gradual incidence than the ascent observed upon omission of either the olfactory or the optic stimulus. The sharp rises obtained in these cases are thus due directly to the omission of a part of the stimulus complex to which the fishes have been trained to respond, and not simply to experimental extinction.

When the olfactory stimulus was omitted a large rise took place in the first response, while when the optic stimulus was absent, the rise in the curve was much greater for the second response than the first. Thus when "smell" was absent the fish were delayed in coming out of compartment *C*, while when light was omitted they were delayed in coming out of compartment *A*. Thus the olfactory stimulus seems to be more important than the visual in connexion with the reaction of coming out of compartment *C*, while the visual stimulus is more concerned with the reaction of coming out of compartment *A*.

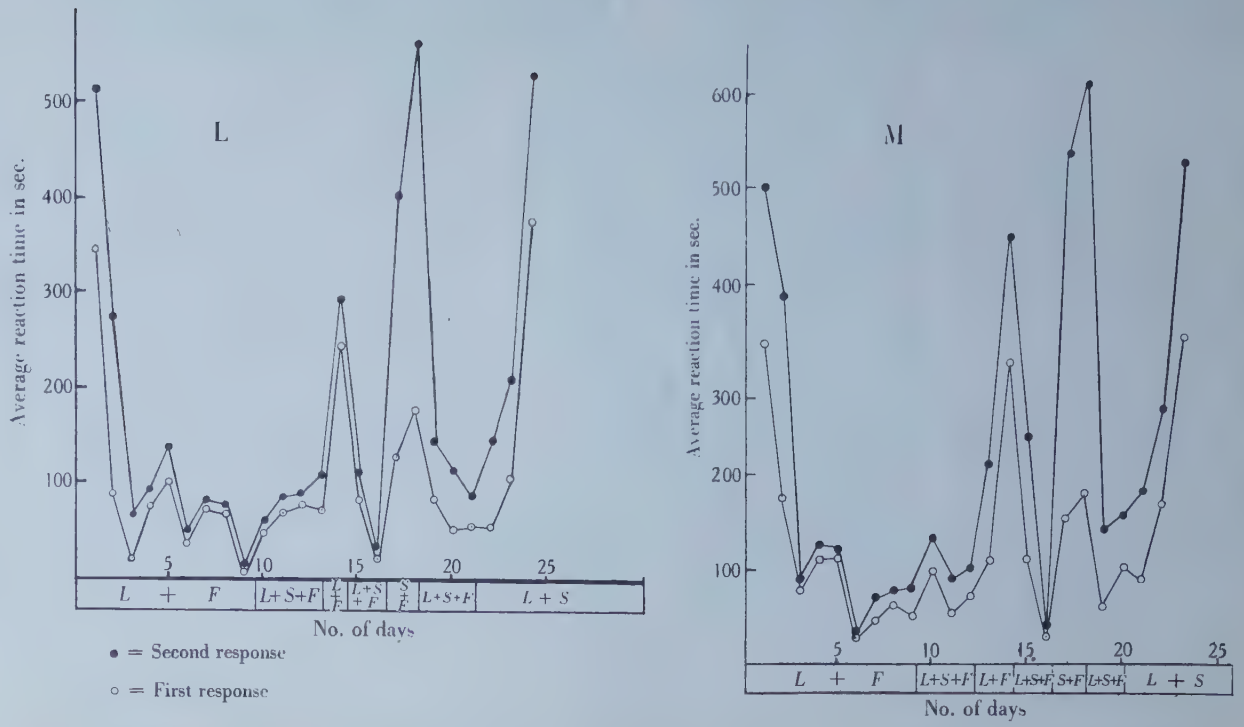

Fig. 4. Learning in response to a combination of visual and olfactory stimuli. Day-average curves of both first and second responses for animals L and M. Changes in the experimental situation made during the experiment shown diagrammatically below each curve. L=light, S=smell, F=food.

SECOND-ORDER OLFACTORY-OPTIC LEARNING

It has thus been shown that there is a mechanism in the brain of the goldfish whereby a learned state can be established to a specific pattern of olfactory and optic stimulation in association with food as "reward".

Animals were then trained in a preliminary experiment to associate an optic stimulus with food as "reward". Then, in a second experiment, these animals were trained to associate an olfactory stimulus with the same optic stimulus as "reward", and no food was given. As the time of reaction to olfactory stimulation declined upon repetition, the light must have been acting as a "substitute reward" as a prelude to feeding.

Experimental procedure. This was essentially the same as in the preceding series of experiments, except as regards the time of presentation of the stimuli. The animals were first trained (first-order situation) to make both first and second responses to the illumination of the disk in compartment *A*, food being given at the completion of the second response. Then the animals were presented with the second-order situation, that is to say first amyl acetate in compartment *C* with the disk being illuminated as soon as they came out into compartment *A*, and then removal from the tank before they could pass through into compartment *B*. No food was given in any trial in the second-order situation.

Results. Five fishes were used. The day-average curves for the complete experiment are shown in Fig. 5, and curves illustrating the course of the second-order learning, plotted from the reaction times recorded only in the trials in the second-order situation, are shown in Fig. 6.

After each fish had been trained in the first-order situation (i.e. with the light only) until a good response was obtained, it was trained for 2 days in the second-order situation. Then a return was made to the first-order situation for 3 days to reinforce the first-order learning. The animals were then trained in the second-order situation for a further day and so on.

The curves of Figs. 5 and 6 show that the first trials in the second-order situation were accompanied by a large rise in the curve of the first response (the second response was of course not recorded in second-order trials, as the fish were not allowed to go into compartment *B*). During succeeding trials, however, the response dropped steeply down, and the curves of Fig. 6 have the form of learning curves. It is necessary to emphasize that in no second-order trial was food given. In all cases optic stimulation was given as a "substitute reward". Also it seems unlikely that the fishes were simply learning to come out of *C* as a response to a distasteful stimulus (amyl acetate) since:

(*a*) In no trial did they come out in the preliminary waiting period.

(*b*) An equally distasteful stimulus was provided outside compartment *C* by almost immediate capture in compartment *A*. This should, if anything, cause the fishes to learn to remain in compartment *C*.

The only alteration in the constant situation was that provided by the illumination of the disk as soon as each fish left compartment *C*. The illumination of the

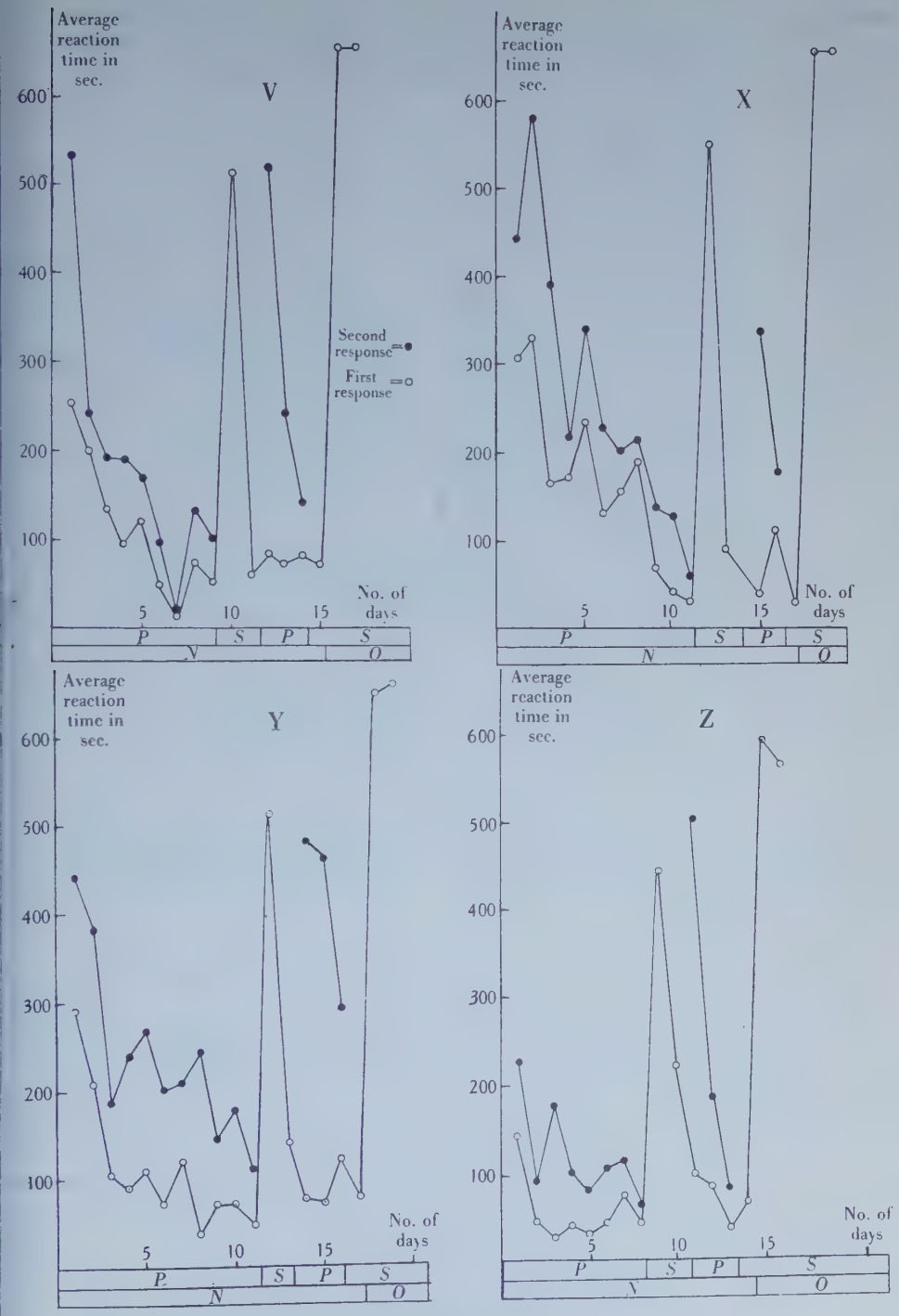

Fig. 5 *a*. (For legend see Fig. 5 *b*.)

disk, by virtue of its association with food in the first-order training, can be substitute for feeding in the subsequent olfactory training. This is an effect comparable to second-order conditioned reflexes (see Pavlov, 1927).

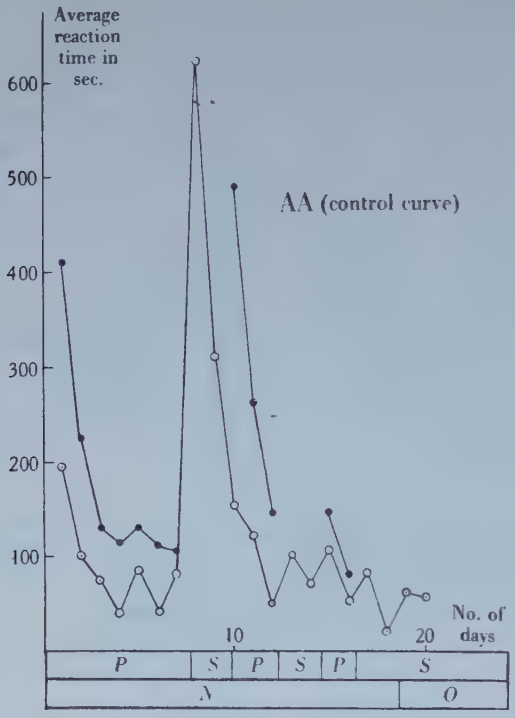

Fig. 5*b*.

Fig. 5*b*. Second-order olfactory-optic learning. Day-average curves of both first and second responses for the complete experiment in animals V, X, Y, Z, AA. Changes in the experimental situation, and the time of operation, shown diagrammatically below each curve. *P* = first-order situation, *S* = second-order situation, *N* = normal, *O* = operated.

THE EFFECT OF TECTAL LESIONS UPON SECOND-ORDER LEARNING

The tectum was operated upon in four animals which had been trained in the second-order situation. In one animal cuts were made under the edge of the optic tectum anteriorly, in order to damage the backward projections from the forebrain into the tectum; in the other three animals large areas of the tectal roof were removed. Fig. 7 is a chart prepared from study of the histological material provided by these operated animals, showing the extent of the lesions made. It will be seen that even in the most extensive lesions large portions of the tectal roof remained intact laterally. In a fifth animal a large flap of the skull and meninges was removed without damage to the underlying tectum. This was used to control the possibility of any disturbance observed being due to the exposure of the nervous tissue, and not to the actual cuts made.

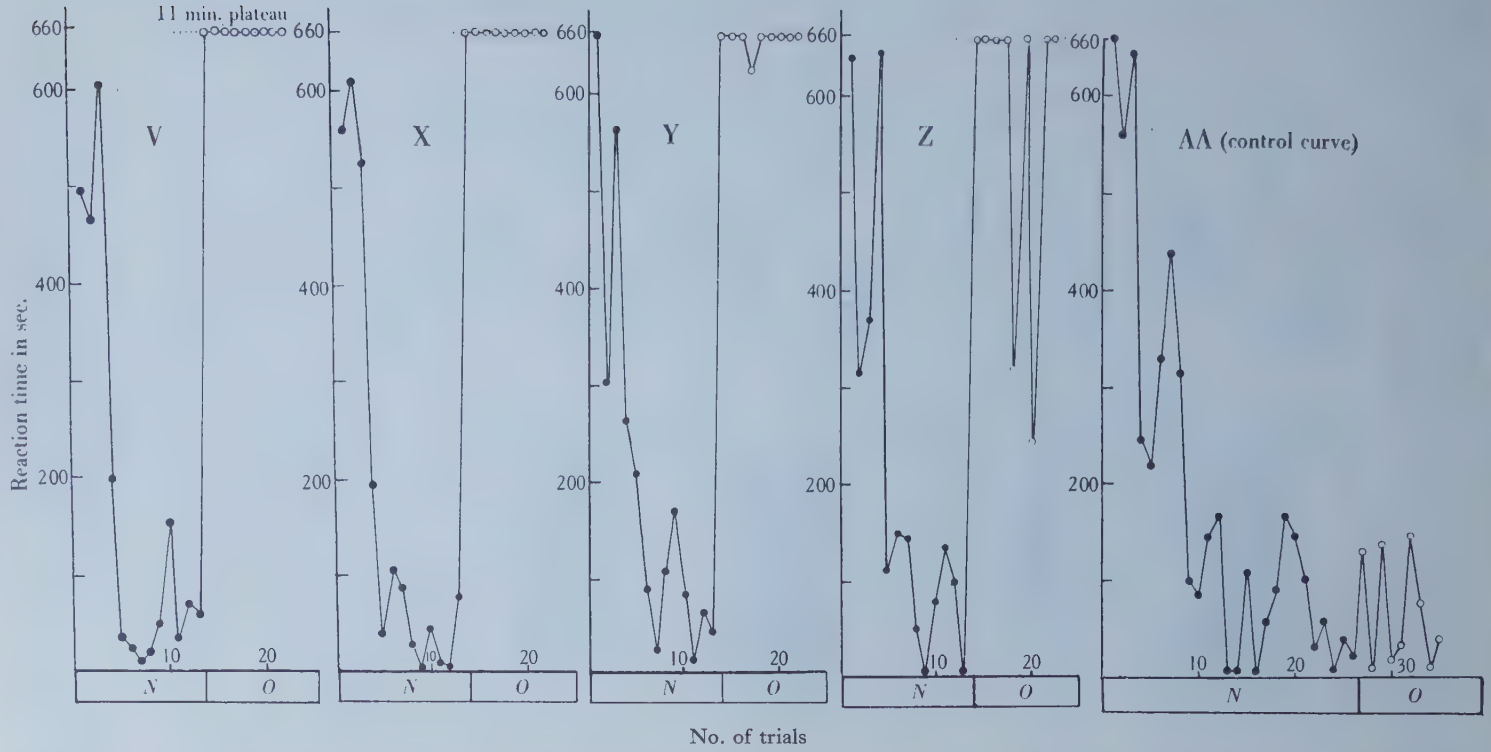

Fig. 6. Second-order olfactory-optic learning. Curves plotted from the second-order trials alone in animals V, X, Y, Z, AA. Time of operation shown (diagrammatically below each curve. *N* = normal, *O* = operated.)

Figs. 5 and 6 show the form of the second-order learning curves for these five animals before and after operation. In all cases except the control animal, there was maximal disturbance of the learned state upon operation. The control curve

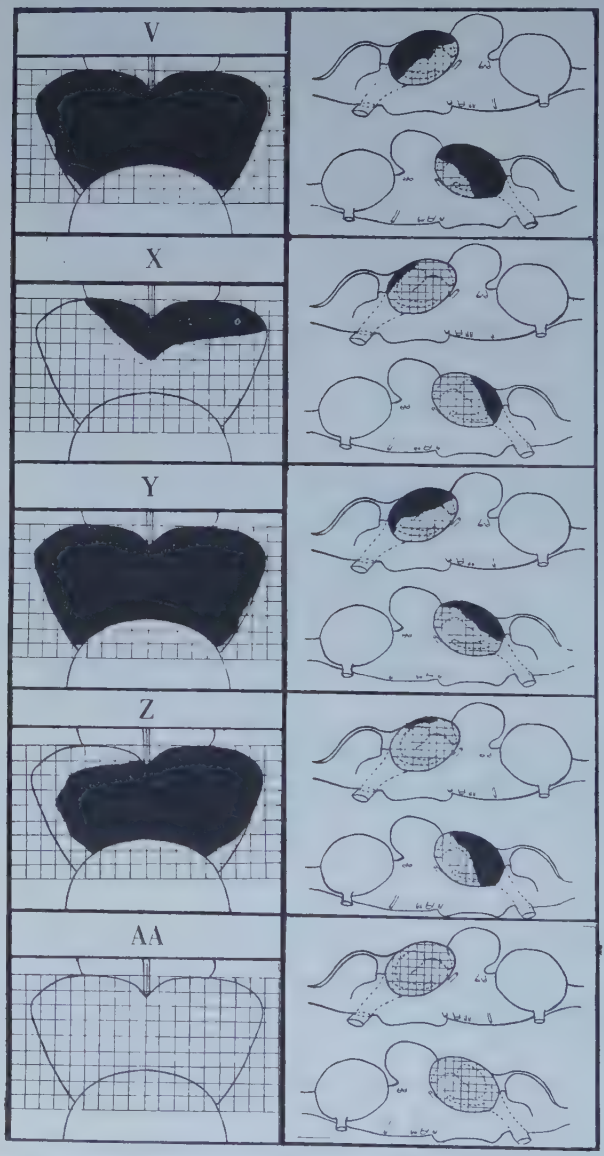

Fig. 7. Chart of the tectal areas removed in animals V, X, Y, Z, AA. The extent of the lesions are shown black.

does not show this disturbance, and during nine further trials in the second-order situation no noticeable disturbance appeared. Thus the disturbances of the curves observed were due to the tectal lesions, and not to the exposure of the brain through the open skull roof, since they only appeared when the optic tectum was damaged.

The swimming movements of the operated animals were essentially normal. Thus the delay in performance of the learned response was due to a disturbance of the learned state, and not to interference with mechanisms responsible for swimming or orientation.

DISCUSSION

From these experiments it is clear that the goldfish is capable of more complex types of learning than the simple conditioned reflex. Fishes which have been trained to associate feeding in a certain compartment of the experimental tank with the display of an illuminated disk in another compartment can then be taught to associate a "smell" in yet a third compartment with the illuminated disk. Thus there exists in the Teleost brain a higher nervous centre where:

(1) As a result of repetitive optic stimulation in association with the presence of food, a state of activity is set up so that optic stimulation thereafter calls forth a reaction previously unconnected with it.

(2) The activity produced by (1) provides the opportunity for a further state of activity as a result of repetitive olfactory stimulation in association with the newly "significant" optic stimulation, whereby an olfactory stimulus can call forth a reaction previously not elicitable by it.

Extirpation of large areas of the sheet of tissue forming the roof of the optic lobes, or cuts made at their anterior border, abolish the capacity of olfactory stimulation to elicit this reaction. At first it might appear that this is due to a disturbance of the "state" in the olfactory system as a result of the interruption of an essential flow of impulses from the optic lobes. But in the Teleost there is no proof of the existence of forward projections from the optic lobes into the olfactory centres (see Kappers *et al.* 1936), and thus the flow of impulses interrupted must be from the telencephalon into the optic lobes, and not vice versa. The change of "state" during second-order learning must therefore take place in the optic lobes and not in the forebrain, and some of the essential pathways involved enter the tectum at its anterior border, since comparatively local cuts made in this region, as well as extirpation of areas of the tectum, interfered with the learned state.

Young (1938), on the basis of the demonstration of specific cyclical nervous connexions in the central nervous system of *Sepia*, showed how they could be used to explain some of the phenomena of learning on the known facts of nerve physiology (for summary of previous theories see Lashley, 1934). Lorente de Nó (1938) has drawn attention to the importance of cyclical connexions amongst internuncial neurons, and points to the existence, within masses of nervous tissue, of cells whose branches do not extend outside the mass, and whose discharge may serve merely to change the threshold to stimulation of other cells within the mass (e.g. Golgi Type II cells in the mammalian cortex; see Lorente de Nó in Fulton, 1938). In the teleostean optic tectum the actual cell connexions have a certain general similarity to those of the mammalian isocortex (see Kappers *et al.* 1936). Afferent fibres pass close to the surface in a superficial neuropil, while the third layer from the surface contains large pyramidal cells which constitute the cell bodies of the main efferent

tracts (see Fig. 8). Possible cyclic connexions of several different types are present and learning can be conceived as a complex of activity in the tectum, maintained by these cycles, and producing a constant state of facilitation of the efferent cells to various specific patterns of afferent impulses. Thus a pattern of afferent stimulation including, say, optic and visceral sensory impulses, could be conceived as setting up cyclic activities within the tectum which would maintain efferent thresholds at such a level that subsequent afferent stimulation would be able to produce a motor response of which it was not previously capable. Visual learning would consist in the production of a constantly maintained facilitation of specific tectal efferents to a specific pattern of optic stimulation, by its association with concurrent

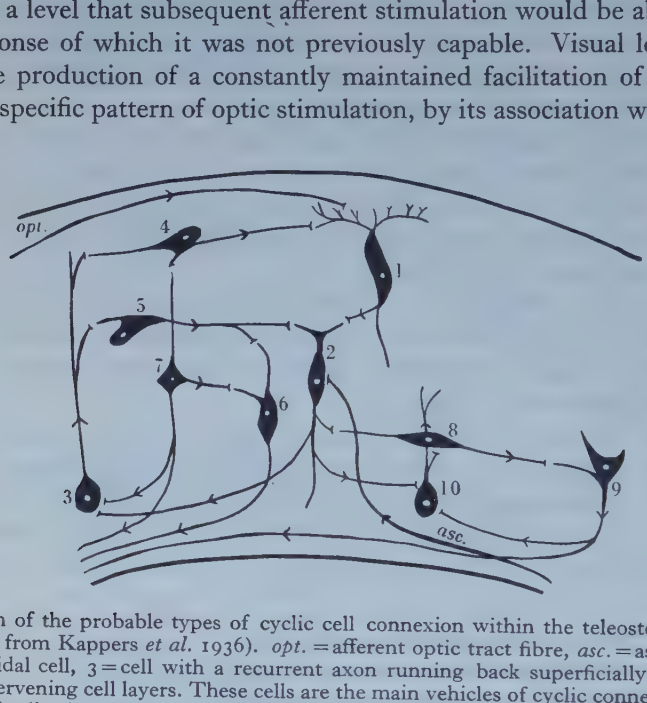

Fig. 8. Diagram of the probable types of cyclic cell connexion within the teleostean tectum (very much simplified from Kappers *et al.* 1936). *opt.* = afferent optic tract fibre, *asc.* = ascending afferent fibre, 7 = pyramidal cell, 3 = cell with a recurrent axon running back superficially and giving collaterals to all intervening cell layers. These cells are the main vehicles of cyclic connexion. Following the excitation of cell 1 by optic tract impulses, the following cycles of activity can be set up under appropriate conditions: 1, 2, 3, 4, 1: 1, 2, 3, 4, 7, 3, 4, 1: 1, 2, 3, 5, 2: 1, 2, 8, 9, 10, 8: 1, 2, 3, 5, 2, 8, 9, 10, 8. The anatomical bases for cycles of even greater complexity are present.

visceral sensory impulses. Second-order olfactory learning would consist in the cyclically maintained facilitation of efferent cells to olfactory stimulation, by its previous association with a specific pattern of optic stimulation, to which there was already a facilitation of the tectal efferents through earlier association with visceral sensory stimulation.

The difficulty in any such theory is to understand the mechanism whereby a specific reaction is given to a specific pattern of stimulation received anywhere on the sensory surface. In visual learning the fish may be in a different part of compartment *A* (see Fig. 1) each time the disk is illuminated, and it must be rarely that its image is projected on to the same area of the retina in successive trials. Definite areas of the retina bear a constant relation to definite areas of the tectal surface (Kappers *et al.* 1936), and thus different parts of the tectum must be excited by the same pattern of stimulation in different trials. Both visual and second-order

olfactory learning must thus appear as a local response of a very large area of the optic tectum.

Ablation of areas of the tectum is not likely to give a great deal of information on the actual neural mechanism involved in visual and second-order olfactory learning. In *Sepia* (Young, 1938) the cyclic mechanism involves large numbers of cells with similar connexions, lying in two masses in different lobes of the supra-oesophageal ganglion, so that cuts can be made between the lobes which interrupt the cycle, without damage to neurons concerned with other functions (Sanders & Young, 1940). In the teleostean tectum the whole mechanism lies within the thickness of the tectum, and such a complex system of interconnected neurons exists, that present operative methods are not sufficiently accurate to interrupt specific cycles, and at the same time to exclude other variables. Yet a great complexity of patterns of activity must be a constant feature of these higher centres during the life of the animal, and the roof of the midbrain, in the Teleostei, must occupy a position in relation to the regulation of "higher" activities, analogous to that of the cortex of a mammal.

SUMMARY

1. The goldfish (*Carassius auratus*) learns to swim to concealed food when an illuminated disk is presented.

2. The presentation of an olfactory stimulus at the same time as the optic stimulus, during reinforcement of this visual learning, results in the addition of the olfactory stimulus to the stimulus complex necessary to call forth the learned reaction.

3. Five animals learned to react to a situation in which—after preliminary training with an optic stimulus with food as reward—amyl acetate was given as olfactory stimulus and the optic stimulus as reward. This effect is termed second-order learning. It is comparable to second-order conditioned reflexes.

4. In four animals trained in this way operations involving removal of large areas of the optic tectum, or cuts made at its anterior border, caused disturbances in the second-order learning.

5. There exists, therefore, in the Teleostean optic tectum a mechanism capable of second-order olfactory-optic learning.

6. The connexion between the telencephalic primary olfactory centre and the tectum involved in this learning passes into the tectum at its anterior border, since cuts made in this region interfered with the learning while leaving a great deal of the tectum intact.

The author is greatly indebted to Mr J. Z. Young for his helpful criticism and advice throughout the course of the work.

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THE EFFECT OF CERTAIN ENVIRONMENTAL FACTORS ON THE GROWTH OF BROWN TROUT (*SALMO TRUTTA* L.)

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(*Received* 16 *September* 1940)

(With Four Text-figures)

INTRODUCTION

The factors influencing the growth of salmonid fishes may be divided into two groups—physico-chemical and biotic. In the first group light, water temperature and the chemical constitution of the medium are probably the most important: in the second group a single factor—the food available to the fish—would appear to be of greatest significance.

Seasonal variations in the growth rate of salmonid fishes in any one environment have long been recognized, but it is only recently that the specific factors responsible for these variations have been investigated. Allen (1940) studied such variations in salmon parr (*Salmo salar* L.) in the field and concluded that water temperature is probably the basic factor in determining whether or not growth takes place. It is clear, however, that no definite conclusions can be drawn as to which of the various factors mentioned above is the basic one until these seasonal variations have been investigated under controlled conditions. Unfortunately the greatest difficulty has been experienced in effecting these variations in the laboratory. Pentelow (1939), for example, working with brown trout (*S. trutta* L.), could detect no significant seasonal variations in growth rate. Appropriate aquarium facilities were, however, available in the Natural History Department, Aberdeen University, and it was decided to attempt a determination of the basic growth factor under experimental conditions. The results of this work are described in the first part of this paper.

Besides seasonal variations in a specific environment absolute differences in the growth rate of salmonid fishes from different environments are known to exist. In the British Isles these differences can best be demonstrated by comparing the size attained by brown trout in "peaty" hill streams with that reached by the same species in lowland chalk streams. In the former environment even four- to five-year-old fish may reach only 4 oz. (0·11 kg.) in weight: in the latter a weight of 3 lb. (1·36 kg.) at three to four years of age is by no means exceptional. The specific factors responsible for these differences are at present rather more obscure than those concerned in seasonal variations, and it is only possible to assess them in the broadest of terms "acid" and "alkaline" waters, "hard" and "soft" waters.

Southern (1932, 1935) studied the growth of brown trout in certain "acid" and "alkaline" waters in Eire and showed that in "alkaline" lakes and rivers on limestone rocks fish are large, grow rapidly, become sexually mature late and are noted for their longevity: in "acid" water bodies derived from Old Red Sandstone covered with peat on the other hand, fish are small, grow slowly, become sexually mature early and are short lived. Subsequent studies on other Irish waters confirm Southern's results (Frost, 1939). In Britain, however, some exceptions have been found to this general rule. In Sutherland and Caithness "acid" lochs have been found to yield trout of 3 lb. or more (*Salmon and Freshwater Fisheries Reports*, 1936, 1937). In the Lake district of England large differences in the growth of brown trout from uniformly "acid" waters have been shown to exist (Swynnerton & Worthington, 1939). Raymond (1938) on the other hand substantially confirmed Southern's findings by correlating the growth of trout in various British waters with the neighbouring geological strata. It should be pointed out, however, that in this latter work no chemical analyses of the various waters dealt with are given: it is thus somewhat speculative to speak of any correlation between the constitution of the waters (as opposed to the surrounding rocks) and the degree of growth achieved. In spite of the above exceptions British data are on the whole not at variance with Southern's conclusions that trout grow large in "hard" (alkaline) waters and remain small in "soft" (acid) waters.

The specific factor responsible for this size difference was for a long time thought to be differences in food supply in the two environments. Southern (1932, 1935), however, has shown that in the "acid" waters of Lough Atorick in Eire where the trout never exceed 6 oz. (0·17 kg.) in weight the food supply is apparently in excess of the requirements of the fish. In view of this Southern suggested that the factor responsible might well be the difference in chemical constitution of the two types of water.

"Hard" and "soft" water differ in their chemical constitution in a number of important respects: the main differences are shown in Table 1. It is clear that for

Table 1. *Composition of "hard" and "soft" fresh waters (after Baldwin)*

Figures denote mg. per 100 c.c.

Water	Na	K	Ca	Mg	Cl	SO_4	CO_3
Hard	2·1	1·6	6·5	1·4	4·1	2·5	11·9
Soft	1·6	—	1·0	0·05	1·9	0·7	1·2

all the ions mentioned, "hard" water contains a greater concentration than "soft". The importance of the calcium ion in various biological processes has, however, long been recognized and it has therefore been suggested that the difference in concentration of this ion, either alone or in combination with other ions, is the basic factor responsible for the size difference of fish living in the two types of water. This suggestion has been borne out to some extent by the work of Tunison & McCay (1931, 1935) in the U.S.A., who showed that a large proportion of the calcium requirements of brook trout (*Salvelinus fontinalis* Mitchell) can be obtained

lirect from the water. Apart from some short term experiments described in the *Salmon and Freshwater Report*, 1936, however, no attempt has been made to study he effect of different calcium concentrations on the growth of salmonid fishes. It ppeared desirable, therefore, to undertake some experiments in this connexion: he results obtained are described in the second part of this paper.

MATERIALS AND METHODS

Yearling brown trout (*Salmo trutta* L.) were used in the work. They were btained from the Howietoun and Northern Fisheries, Stirling, in February 1939 14 months after hatching) and kept in the laboratory throughout the period of the xperiments.

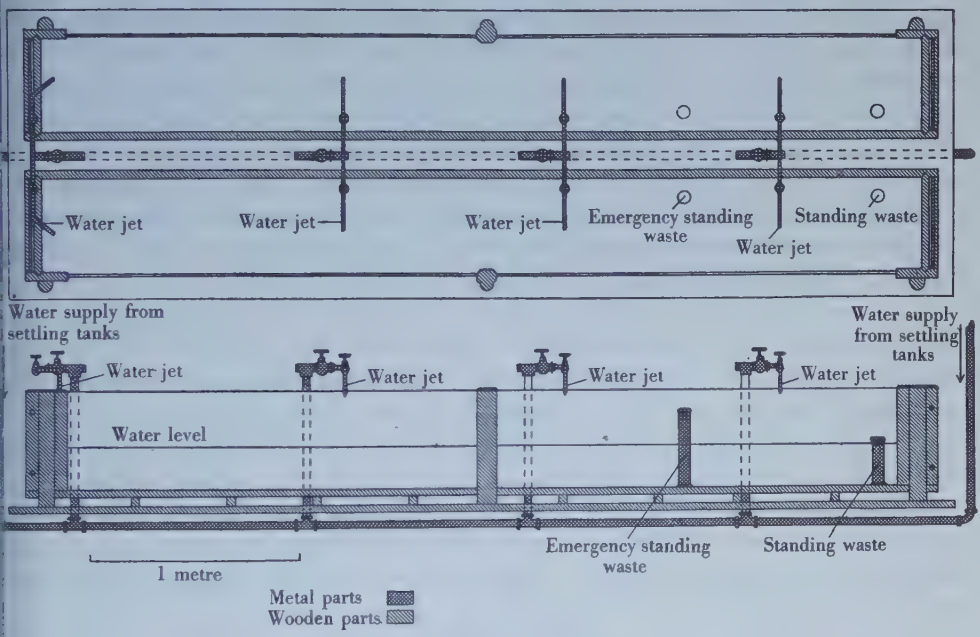

Fig. 1. The aquarium tanks.

Two identical aquarium tanks were used to accommodate the fish. These were ectangular in shape (14 ft. 3 in. × 1 ft. 9 in. × 1 ft. 8 in. [4·34 m. × 0·53 m. × 0·50 m.]) nd constructed of $1\frac{1}{2}$ in. (3·81 cm.) teak fronted with $\frac{3}{8}$ in. (0·96 cm.) plate glass long one of the longer sides. The tanks were arranged back to back underneath a arge skylight to ensure adequate and equal illumination. Each tank was supplied with four water jets arranged more or less equidistantly along its length. All water ets were fed from settling tanks located in the roof, in which the water level was kept constant by ball valves: in this way the quantity of water passing to each of the anks was kept constant and equal. The water level in the aquarium tanks was maintained at 8 in. (20·32 cm.) from the bottom by means of a standing waste. An mergency standing waste was also employed to avoid flooding in the event of the

normal waste becoming blocked. In order to prevent fish escaping, each tank was covered with $\frac{3}{8}$ in. wire mesh arranged in four sections separated by the water inlets. The detailed structure and arrangement of the tanks and water supply are shown graphically in Fig. 1.

On arrival in the laboratory the fish were divided into two batches of twenty-four each, transferred to the tanks described above and left for a period of 6 weeks to settle down. During this period no experimental readings were taken.

The effect of different calcium concentrations on the growth of the fish was studied by adding, at a constant rate, drops of a concentrated solution of calcium chloride (0·55 kg. per litre of solution) to the water in one of the tanks and using the other tank containing untreated tap water as a control: the chloride was used because of its cheapness and great solubility. Calcium determinations by a method based on that of Kramer & Tisdall (1921) as modified by Clark & Collip (1925) were made on the water of both tanks at periodic intervals. That of the control tank remained very constant at 0·4 mg. calcium per 100 c.c. water (the calcium content of a very "soft" water). In the calcium tank, although small variations in the rate of dropping of the calcium chloride solution caused corresponding fluctuations in the total calcium content of the water, this was maintained more or less constant at 5 mg. calcium per 100 c.c. water (the calcium content of a typical "hard" water). Other than this difference in the dissolved calcium content there was no distinction between the experimental treatment of the two tanks.

The water temperature of both tanks was recorded daily. No significant difference between the two tanks could be detected at any one time. The hydrogen-ion concentration of the water was estimated periodically during the course of the experiments: it was found to remain fairly constant at *p*H 7·0.

The fish were found to survive captivity reasonably well. Throughout the course of the experiments the mortality was six fish from the control tank and four fish from the calcium tank. It was found necessary, however, to treat the tanks with a solution of potassium permanganate once every two or three weeks to prevent the growth of fungus: this procedure was especially important during the summer months.

The fish were fed daily on a diet made up of minced liver, chopped earthworms and *Daphnia* suspensions: both tanks received the same quantities of food. Prior to each feeding all food left over from the previous day, together with excreta, were removed from the tanks.

The growth of the fish was recorded as follows. The fish from each tank were weighed collectively and their length measured individually once a month from 3 April 1939 to 7 March 1940. They were removed from the tanks with a hand net, the majority of the water allowed to drain off, and transferred to a previously weighed can containing sufficient water to accommodate the fish without overcrowding. This was then weighed to the nearest 0·5 g. the original weight of can and water subtracted and the total weight of fish recorded. The fish were then removed from the can one by one, placed on a towel saturated with water and their length measured to the nearest 0·5 cm. As soon as this latter measurement was

completed the fish were returned to the main tank. Readings were expressed as the average weight or length of a single fish.

In an attempt to corroborate these growth readings scale samples were taken from six fish in each tank at the end of the experiments. The scales were macerated for 14–21 days in tap water, after which they were scrubbed clean with a stiff camel-hair brush, washed thoroughly in distilled water and mounted in "Euparal" for microscopic examination.

The effect of different concentrations of calcium in the water on the amount of this element stored in the body of the fish was studied by making calcium analyses of the vertebrae of the fish used for scale samples. The analyses were done as follows. The fish were killed by guillotining and the backbone roughly separated from the carcass. Complete separation of the individual vertebrae from the surrounding tissue was effected by the method of Subrahmanyan *et al.* (1939).

After disarticulation was complete the vertebrae were washed several times with hot distilled water. A number (weighing *c.* 0·1 g.) were then roughly dried on filter paper, transferred to a previously weighed crucible and dried overnight at 40° C. After recording exactly the dry weight of each sample the vertebrae were ashed to "whiteness" in the usual way. When cold the material was digested with a small quantity of 2*N* HCl, transferred to a 100 c.c. flask and made up to the mark. Calcium analyses of aliquot portions of this solution were then made by the method referred to above: results were expressed in milligrams calcium per gram of dried bone.

EXPERIMENTAL

One batch of fish was used to investigate the role of water temperature and food supply in effecting seasonal variations in growth. The other batch was employed to determine the effect of higher concentrations of dissolved calcium on growth, the first batch being used as a control.

(1) *Water temperature and food supply*

Although either of these two factors might be the basic one in controlling seasonal variations in the growth of trout, in view of preliminary observations both in the field and in the laboratory indicating the importance of the water temperature, it was decided to investigate this factor first. Accordingly the water temperature was not controlled and varied with the season of the year. The food supply, on the other hand, was maintained always in excess of the requirements of the fish, so that this never constituted a limiting factor.

A general idea of the temperature variations during the experiments can be obtained from the twice monthly readings given in Table 2 and summarized in Fig. 2. The extremes recorded were 2·5° C. on 21 January 1940 and 17·1° C. on 28 August 1939.

The amount of food given per week was expressed in arbitrary units—grams of minced liver, grams of chopped earthworms and measures (300 c.c.) of *Daphnia* suspensions. During the very cold weather from 26 January to 15 February 1940 *Daphnia* suspensions could not be obtained. They were replaced by either Tubificids

Table 2. *Variations in water temperature during experiments*

Date	Water temp. ° C.	Date	Water temp. ° C.
15. iv. 39	7·6	15. x. 39	10·6
1. v. 39	8·2	1. xi. 39	8·0
15. v. 39	10·3	15. xi. 39	8·3
1. vi. 39	13·2	1. xii. 39	6·2
15. vi. 39	14·7	15. xii. 39	6·0
1. vii. 39	14·6	1. i. 40	3·8
15. vii. 39	14·4	15. i. 40	4·3
1. viii. 39	16·0	1. ii. 40	2·8
15. viii. 39	16·4	15. ii. 40	3·2
1. ix. 39	16·9	1. iii. 40	4·3
15. ix. 39	15·1	15. iii. 40	5·0
1. x. 39	12·2		

or Enchytraeids: the amounts given were expressed in terms of *Daphnia* measures 20 g. of the worms being taken as equivalent to 1 measure of *Daphnia* suspensions. In order to maintain the food supply in excess the amounts given were progressively increased from 1 June 1939 to 15 August 1939, after which they were maintained constant at the level reached on the latter date (Table 3, Fig. 2).

Table 3. *Food given during experiments*

Period of experiment	Food given (units)
3. iv. 39–31. v. 39	31
1. vi. 39– 4. vii. 39	51
5. vii. 39– 9. vii. 39	141
10. vii. 39–26. vii. 39	206
27. vii. 39–14. viii. 39	221
15. viii. 39– 7. iii. 40	301

Table 4. *Growth of trout in Aberdeen tap water (calcium content 0·4 mg./100 c.c.) over period April, 1939 to March, 1940*

Date	Average weight of single fish grams	Average length of single fish cm.	Date	Average weight of single fish grams	Average length of single fish cm.
3. iv. 39	8·2	9·1	5. x. 39	54·8	16·2
2. v. 39	9·4	9·3	8. xi. 39	68·0	17·3
5. vi. 39	10·7	9·7	5. xii. 39	73·1	18·0
7. vii. 39	15·1	10·7	5. i. 40	76·0	18·3
4. viii. 39	24·6	12·2	5. ii. 40	75·6	18·4
4. ix. 39	38·0	14·1	7. iii. 40	80·0	18·8

The growth of the fish both in weight and length is given in Table 4 and summarized in Fig. 2. It will be seen that the shape of the two curves (weight and length) is more or less identical and thus they confirm one another. During the spring (April and May) the growth achieved is small: during the summer it rapidly increases, reaching a maximum in early autumn (September) after which it gradually falls off. In the winter months little growth takes place: during January there is actually a loss in weight.

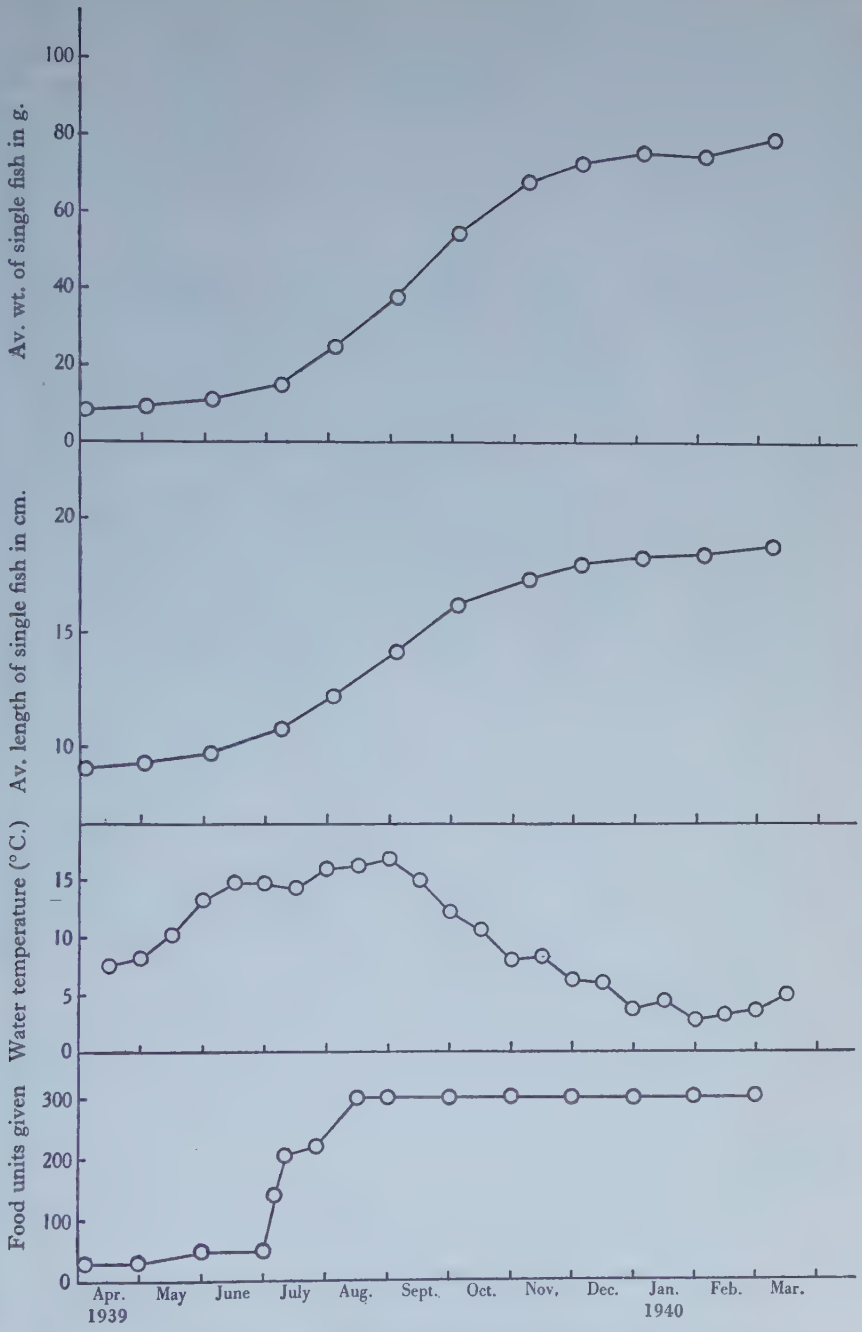

Fig. 2. Growth in weight and length, the water temperature and the food supply during the period April 1939 to March 1940.

The rapid increase in growth during the summer is correlated with a large increase in the food intake which reaches a maximum about August. Similarly the diminution in growth during the winter months is associated with a corresponding decrease in food consumption as shown by the progressively larger amounts of surplus food found in the tanks after feeding. Allen (1940) and Frost & Went (1940) found from an examination of the scales of salmon parr that a falling off in growth occurred during the period July–August: no such diminution was observed in the experiments described above.

The relation between the water temperature and the degree of growth achieved is significant. With rising water temperatures during spring and summer there occurs a rapid increase in growth: with falling water temperatures during autumn and winter growth decreases. The exact nature of this relation was determined by calculating the rate of growth (grams per gram of starting weight) for each month and the corresponding average water temperature (average of daily readings). The results are given in Table 5 and shown graphically in Fig. 3. In the graph the points have been separated into two series—one representing the growth rate/temperature relation during a period of rising water temperature, the other the same relation during a period of falling water temperature. The readings for February 1940 have been omitted from the graph as these constitute the beginning of another rising temperature series. It will be seen that the relation is different in the two series: in the rising temperature series the relation is roughly hyperbolic, in the falling temperature series, linear. Thus the growth rate of fish with a low temperature history increases slowly with rising water temperature; on the other hand the growth rate of fish with a high temperature history decreases rapidly with falling water temperature. In the rising temperature series there is no falling off in the curve even at the highest average temperature (i.e. 16·4° C.): it appears therefore that the lethal temperature is considerably above this level. This is in accord with the experimental work of Gardner & Leetham (1914) who found the upper limit of temperature to be 25° C. and with the field observations of Embody (1921) in the U.S.A. and Phillips (1929) in New Zealand who recorded brown trout at temperatures of 83° F. (28·3° C.) and 77° F. (25·0° C.) respectively.

From the above results it is clear that the seasonal variations observed were brought about fundamentally by variations in water temperature. It should be noted, however, that no attempt was made to control the illumination of the tanks; it is possible, therefore, that the variation in light intensity which was similar to the variation in water temperature (high in summer, low in winter) may have been the responsible factor.

The broken line in Fig. 3 indicates approximately the general relationship between growth rate and water temperature, both series of points being included. It will be noticed that this line cuts the abscissa at 6° C. indicating that this is the critical temperature below which growth does not occur. It is interesting to note that Allen (1940) concluded from field observations on another salmonid (parr of *Salmo salar*) that 7° C. represented the critical temperature in that species. The similarity between the two temperatures is significant.

Table 5. *Growth rate of trout in Aberdeen tap water (calcium content 0·4 mg./100 c.c.) at various water temperatures*

Month	Growth rate g. per g. of starting weight	Average temperature ° C. (mean of daily temperatures)
April 1939	0·15	8·0
May	0·16	10·6
June	0·54	14·8
July	1·16	15·1
August	1·63	16·4
September	2·05	14·3
October	1·61	9·6
November	0·62	7·4
December	0·35	5·3
January 1940	−0·05	3·2
February	+0·54	3·5

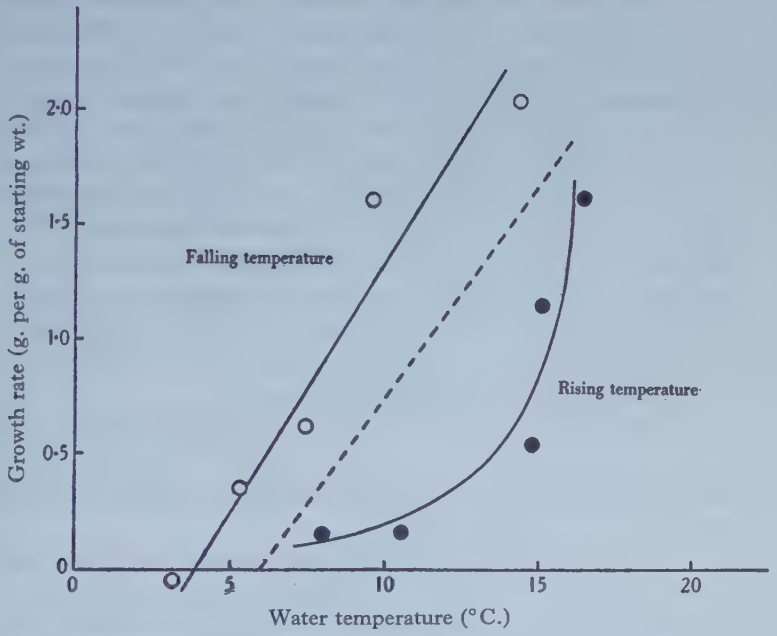

Fig. 3. Growth rate of trout in Aberdeen tap water (calcium content 0·4 mg./100 c.c.) at various water temperatures.

Under natural conditions, however, the basic influence of water temperature on growth is modified by the available food supply. When the former is below the critical temperature little or no growth can occur however great the available food supply: when above, growth is limited by the available food supply. Temperature determinations by the author in the Aberdeenshire rivers Dee and Don (unpublished) show that the water temperature remains below 6° C. for about six months of the year (from the end of October to the end of April). Similar determinations by Butcher *et al.* (1937) in the River Tees (Yorkshire and Durham) and by Allen (1940) in the River Eden (Cumberland and Westmorland) show this period

to be a little less (November to March). For approximately 50% of their lif therefore, trout in rivers in Scotland and Northern England are below their critic temperatures and growth is at a standstill: during the rest of their life wat temperature is above the critical value and growth is only limited by the availab food supply.

Scale rings: it is generally assumed that variations in the growth of salmon fishes under natural conditions are reflected in the width of the rings which occ on their scales. Examination of scales from the experimental fish, however, show no variation in ring width even though seasonal variations in growth had u doubtedly taken place. Gray and Setna (1931) found that provided food w abundant no seasonal periodicity in the width of rings occurred in Rainbow tro (*Salmo irideus* Gibbons). Bhatia (1932) working with the same species showed th variations in temperature had no direct effect on the width of the rings: variatio in available food supply on the other hand were found to bring about alternate zon of broad and narrow rings. It is thus clear why no such zones were found on t scales of the experimental fish: no alternating conditions of abundant and deficie nutrition were present—the available food supply was always in excess of t metabolic requirements of the fish. In nature, narrow rings are laid down duri the winter months, indicating a deficiency in the available food supply. Fie observations, however, tend to show that during the winter the invertebrate fau which constitutes the greater proportion of the food of trout (Neill, 1938) present in sufficient, if not ample quantities. It appears, therefore, that through this period this potential food supply is not available to the fish in sufficient quanti to meet its metabolic requirements. The specific factor(s) responsible for th decrease in the availability of the potential food supply are unknown, but it appea probable that the following factors may be involved:

(1) Inactivity of the fish brought about by low water temperatures decreasi area of feeding.

(2) Alteration in the habits of the invertebrate fauna reducing their availabili as food for the fish.

(3) Changes in certain physico-chemical factors, such as water turbidity a light intensity, rendering the capture of food by the fish more difficult.

The above conclusions concerning the limiting factors to growth under natu conditions must therefore be somewhat modified. Both water temperature a available food supply apparently act as limiting factors during the winter month the possible dependence of the latter on the former has been indicated above. the summer, provided that water temperatures do not rise to a lethal level, only t available food supply is limiting. In the absence of data to the contrary, howeve the possibility of a third major limiting factor such as light intensity being involve must not be excluded.

(2) *Calcium content of the water*

The possible importance of this factor in determining the growth of trout different habitats has been indicated earlier in this paper (p. 436). The experimen undertaken to test this hypothesis were as follows. The dissolved calcium conte

Table 6. *Growth of trout in Aberdeen tap water + calcium chloride solution (total calcium content 5·0 mg./100 c.c.) over period April 1939 to March 1940*

Date	Average weight of single fish g.	Average length of single fish cm.	Date	Average weight of single fish g.	Average length of single fish cm.
3. iv. 39	7·5	9·0	5. x. 39	47·1	15·2
2. v. 39	8·7	9·1	8. xi. 39	56·1	16·3
5. vi. 39	9·7	9·5	5. xii. 39	60·4	16·8
7. vii. 39	14·6	10·4	5. i. 40	64·8	17·2
4. viii. 39	23·8	12·0	5. ii. 40	64·4	17·2
4. ix. 39	32·3	13·4	7. iii. 40	69·2	17·6

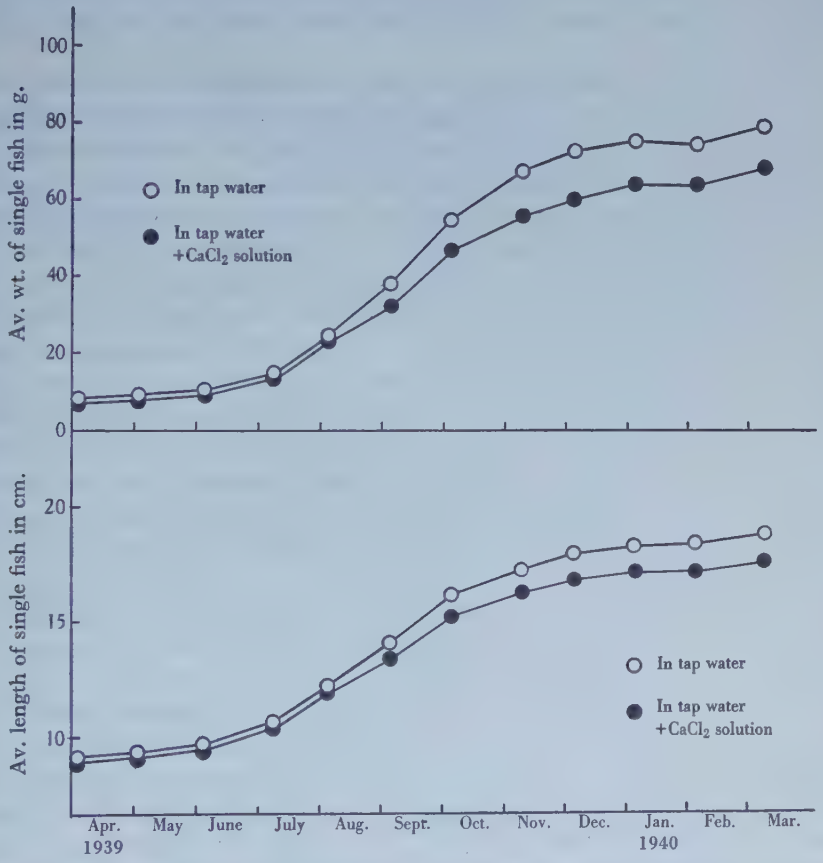

Fig. 4. Growth in weight and length in Aberdeen tap water (calcium content 0·4 mg./100 c.c.) and in Aberdeen tap water plus calcium chloride solution (calcium content 5·0 mg./100 c.c.).

of one of the aquarium tanks was raised at the outset of the experiments (p. 438) and the growth achieved by the fish in this tank compared with that in the untreated tank. The growth of the fish both in weight and length in the former (calcium) tank is given in Table 6. The results are summarized and compared with those from the untreated (control) tanks in Fig. 4.

It will be seen that the growth of the control and calcium series is more or less identical during the first four months of the experiments (April to August 1939). During August, however, the growth both in weight and length of the calcium series begins to fall off (or that of the control series to rise).

This diminution in weight increase is continued during September and October after the latter month the weight increase becomes more or less equal in the two series. The diminution in the increase of length, on the other hand, persists only during September: in October and subsequent months the length increase is approximately the same for both series.

Examination of scales of fish from the calcium tank showed no periodic zones their appearance was more or less identical with those from the control tank. Evidently higher concentrations of dissolved calcium have no effect on the arrangement of scale rings.

It should be noted that the maximum water temperatures occur round the point where the growth curves of the two series commence to diverge (cf. Fig. 2): it appears possible, therefore, that the initial falling off in growth of the calcium series is due to the combined effect of additional dissolved calcium and high water temperatures.

The diminution in growth in water of increased calcium concentration is contrary to the original hypothesis, for if the higher calcium content of "hard" waters is, as was thought possible, responsible for better growth in those waters the raising of the calcium content of a "soft" water to the level of that of a "hard" water should result in an increase of growth. It should be noted, however, that the addition of calcium chloride solution, besides raising the dissolved calcium content also increases the chloride content of the water. It may be, therefore, that the effect observed was that due to the increase in dissolved chloride, the action of increased calcium being masked thereby. It has not yet been possible to test this hypothesis by using another very soluble calcium compound such as calcium nitrate to raise the calcium content and comparing the effect produced with that already found using calcium chloride.

Another explanation of the results is that the calcium level in the control ("soft" water) tank was still too high for it to function as a limiting factor. This supposition is borne out to some extent by an analysis of the calcium content of the bones of fish from the two series. The results are shown in Tables 7 and 8. It will be seen that there is no significant difference between the two series, showing that just as much calcium was stored by the fish in water low in calcium as in water in which the calcium content had been augmented. If this is the explanation it is quite clear that the concentration of dissolved calcium in natural waters is very unlikely to be the factor determining the nature of fish growth in different environments as there are few natural waters with a calcium content lower than Aberdeen tap water.

It appears probable, therefore, that some factor other than the dissolved calcium content is responsible for the differences in trout growth in "hard" and "soft" waters. The nature of the factor is at present unknown, but in its subsequent elucidation it should be borne in mind that these differences in growth in different environ-

Table 7. *Calcium content of trout bone—control series*

Fish no.	Date	Wet weight of fish g.	Length of fish cm.	Dry weight of bone sample g.	Mg. calcium per g. dry bone
1	12. iii. 40	119·5	21·5	0·0673	208
3	16. iii. 40	124·5	22·0	(*a*) 0·0538 (*b*) 0·0510	236* 237*
4	16. iii. 40	51·0	17·5	0·0522	230
5	31. iii. 40	76·5	18·5	(*a*) 0·0557 (*b*) 0·0559	217* 218*
6	31. iii. 40	97·5	21·0	0·0561	223
7	31. iii. 40	28·0	13·5	0·0596	232
					Average 225

Table 8. *Calcium content of trout bone—calcium series*

Fish no.	Date	Wet weight of fish g.	Length of fish cm.	Dry weight of bone sample g.	Mg. calcium per g. dry bone
1	12. iii. 40	151·5	23·0	0·0642	203
2	12. iii. 40	28·5	13·5	0·0632	237
3	16. iii. 40	41·0	15·5	0·0563	227
4	16. iii. 40	107·5	21·5	(*a*) 0·0522 (*b*) 0·0540	228* 224*
5	31. iii. 40	62·5	17·0	(*a*) 0·0557 (*b*) 0·0569	219* 216*
6	31. iii. 40	70·0	18·5	0·0537	214
					Average 221

* Duplicate estimations.

ments may be effected, not by differences in the concentration of any one specific ion, but by departures from the optimum ionic balance brought about by variations in the relative concentration of any of the ions present.

SUMMARY

1. The factors responsible for seasonal variations in the growth of brown trout in any one environment are examined.

2. The probable basic importance of the water temperature is established. A critical temperature of 6° C. below which no growth takes place is deduced.

3. It is shown that the seasonal variations in growth observed do not result in corresponding variations in the width of the scale rings: such variations appear to be the direct result of fluctuations in available food supply.

4. Under natural conditions both water temperature and available food supply apparently act as limiting factors during the winter: during the summer, provided that the water temperature does not rise to a lethal level, only the available food supply is limiting.

5. The possible importance of light intensity as a limiting factor is indicated.

6. Differences in the growth rate of trout from different environments are also considered.

7. The role of dissolved calcium is investigated. It appears unlikely that the amount of dissolved calcium is responsible for the differences in trout growth in "hard" and "soft" waters.

8. It is suggested that such differences may be effected not by differences in the concentration of any one specific ion; but by departures from the optimum ionic balance brought about by variations in the relative concentration of any of the ions present.

I am indebted to Prof. L. Hogben, F.R.S., for his help and advice. To the Fisheries Division of the Scottish Home Department, especially to W. J. M. Menzies and P. R. C. Macfarlane, I would express my sincere thanks.

The investigation was supported by a series of grants from the Development Commission.

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THE PERMEABILITY OF GREGARINE PROTOZOA FROM THE GUT OF THE MEAL-WORM[1]

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(*Received* 10 *September* 1940)

(With Eight Text-figures)

INTRODUCTION

The Gregarine Protozoa, the permeability of which was investigated in these experiments, were taken from the gut of the meal-worm. Since they are without a mouth, any food entering the cytoplasm must do so by penetrating the body surface. This involves the passage of fairly large molecules, and the aim of this research was to find out whether the cell surface was modified in any way to allow the passage of food substances. By application of similar methods to those of Jacobs (1934) and Stewart & Jacobs (1936) it was possible to estimate in absolute units the permeability of *Gregarina* and compare it with that of other cells.

MATERIALS AND METHODS

The permeability was estimated from volume changes in hypotonic and hypertonic solutions. Therefore cells used for experiments had to be symmetrical in order that measurements might be made of their volume and surface area, and they had to be able to withstand, without damage, considerable variations in the external medium.

The species most frequently used was *G. cuneata* Stein, which shows considerable variation of form. The shape of the trophozoite may be seen in Fig. 1. It is

Fig. 1. *Gregarina cuneata* showing trophozoites of an associating pair.

circular in cross-section, and 100–200μ in length and about 50μ in diameter. The cuticle appears to be fairly thick, and when the trophozoites were placed in hypertonic solutions so that exosmosis occurred, it showed longitudinal striations or wrinkling. The deutomerite and protomerite are separated by the inner layer of the ectoplasm. A few experiments were done on *G. polymorpha* (Hamm) which is

[1] Thesis approved for the degree of Doctor of Philosophy in the University of London.

similar but smaller. The trophozoites were used for experiments when they ha associated in syzygy. At this stage the cuticle of each trophozoite remained inta and each could still be regarded as a separate individual. To estimate permeabilit measurements were made on the more posterior of the two associating individual *Gregarina* is very suitable material for a study of permeability for several reason It has a definite shape, a clear outline and photographs well; it can withstan considerable change in concentration and composition of the external mediu without apparent damage; and death is obvious because it leads to rapid and cor tinuous swelling due to the imbibition of water.

On removal from the meal-worms the parasites were placed under a supporte coverslip in an approximately isotonic solution, i.e. 0·7 *M* sucrose. This was slightl hypertonic to the cytoplasm of the parasites; but after a small initial shrinkage th volume of the animal remained constant for periods up to 3 hr. One drop of th irrigating fluid was added to the microscope slide every 5 min. except when th composition of the fluid was changed. Then two drops of the new solution wer added at the moment of change and another a minute later and the irrigation wa continued as before. The parasites were kept in the initial irrigating fluid for a least half an hour, during which time three records of the volume were made Since the volume change was most rapid in the first few minutes after a change i the irrigating fluid, records of the volume were made at intervals of a few second during this period.

The volume was recorded by means of photographs with a microfilm camera. A stage micrometer was also photographed to obtain a measure of the magnificatio Enlarged tracings of the photographs were made by means of a microprojecto These tracings represented a series of longitudinal sections of the trophozoites o known magnification. From them the actual volumes of the trophozoites wer obtained by means of the Amsler integrator (kindly lent by Prof. Levy, F.R.S.E of the Royal College of Science). Imperfect symmetry and errors in focusin probably accounted for fluctuations in volume recorded for the cell when i equilibrium with the external medium.

GREGARINA AS AN OSMOTIC SYSTEM

For purposes of a theoretical treatment of permeability it was necessary to fin out to what extent the cell might be regarded as a dilute solution surrounded by perfectly semipermeable membrane. If the cell were such a system it would b expected to obey Boyle-van't Hoff's law, according to which

$$PV = P_1V_1 = K,$$

where P and P_1 are the internal osmotic pressures and V and V_1 are the corre sponding volumes. Values for PV and P_1V_1 were obtained for *Gregarina* by trans ferring it from 0·7 *M* sucrose solutions to hypertonic solutions of sucrose of know strength. Marked discrepancies occurred between the two values. These dis crepancies might have been due to a leakage of solutes or to the presence of insolubl

[1] I am indebted to the Dixon Fund of the University of London for a grant to purchase this.

substances. That they were not due to a leakage of solutes was shown by the constant volume maintained in 0·7 *M* sucrose solutions and in hypertonic solutions after an initial fall in volume (see sucrose curve). If sucrose were entering or solutes escaping from the cell the volume would not have remained constant.

Experiments with hypotonic solutions were made before the camera was purchased, and the volume was found by making a series of measurements of length and breadth, drawing the parasite to scale and then measuring the volume from the drawing by means of the integrator. This method did not give an accurate record of

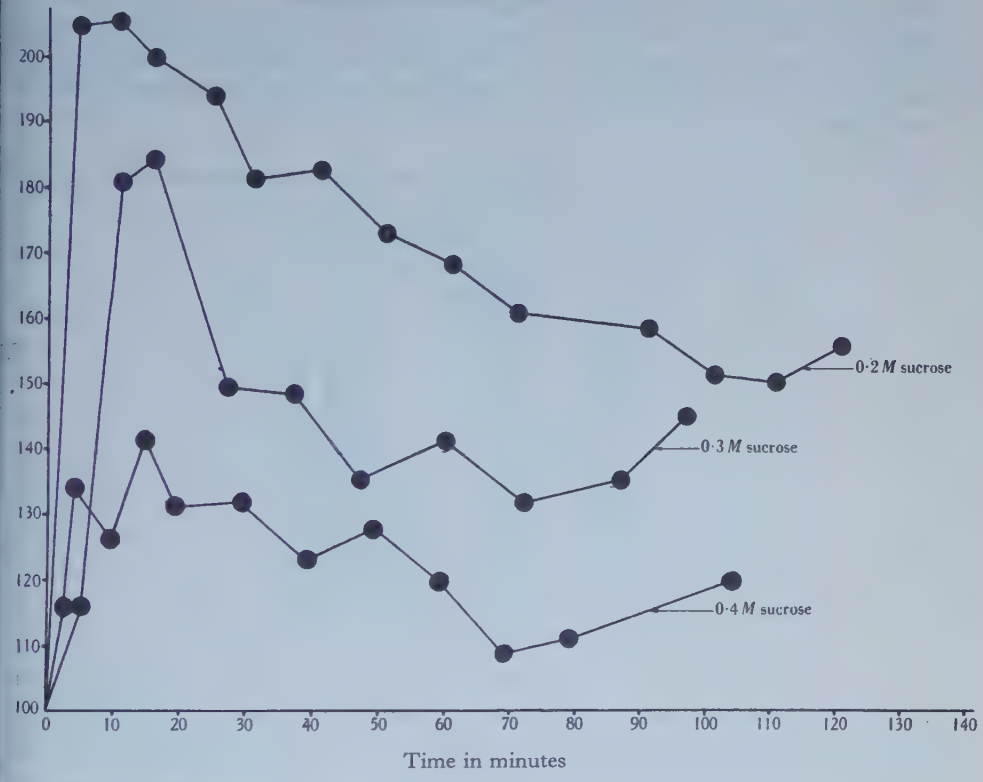

Fig. 2. Changes in volume in hypotonic solutions of sucrose.

the volume when this was changing rapidly, but it indicated roughly the changes taking place. The results are shown in Fig. 2. After the initial rise in volume there was a decrease and the volume finally became constant at a value greater than the initial volume. The initial increase in volume, the decrease and the volume at which equilibrium was established, were all proportional to the concentration of the external medium. The falling off in volume may reasonably be ascribed to a loss of solutes, possibly salts, through the stretched cuticle.

The very granular appearance of the trophozoite suggested that it contained insoluble material. When the cuticle burst the granules emerged in considerable quantities, and they stained deep brown in iodine solution. In Best's carmine they

stained deep pink but, in the control experiment, where the trophozoites were first treated with saliva, the granules disappeared and the cytoplasm remained unstained. This proved the granules to be glycogen, and since glycogen is insoluble its presence would account for discrepancies in the values for *PV*. Giovanola (1934) found *G. cuneata* to be rich in glycogen.

If the total volume of osmotically inactive substances is b, then

$$P(V-b)=P_1(V_1-b),$$

whence $$b=\frac{P_1V_1-PV}{P_1-P}$$ (Lucké *et al.* 1931).

Table 1 shows the values for b obtained in the experiments on *Gregarina*. The fluctuations in these values show no correlation with the concentration of the external medium.

Table 1. *Showing percentage of osmotically inactive substances present in* Gregarina

% of osmotically inactive substances	Strength of external solution *M*
75·2	1·7
82·3	1·7
71·0	1·7
64·8	1·7
83·9	1·7
76·3	1·7
74·0	1·2
83·5	1·2
82·2	1·2
67·7	1·0
70·0	1·0

PERMEABILITY TO WATER

The permeability to water may be calculated from measurements of the volume and surface area before, during, and after shrinkage in a hypertonic medium containing non-penetrating solutes. The formula was derived by Northrop (1927), and is given below in a slightly altered form:

$$\frac{K}{h}=\frac{V_{eq.}}{AP_0V_0t}\left[V_{eq.}\log_e\frac{V_0-V_{eq.}}{V_t-V_{eq.}}+V_0-V_t\right],$$

where K = constant representing the permeability to water,
h = thickness of membrane,
$V_{eq.}$ = volume of osmotically active substances in the cell when equilibrium has been established,
V_t = volume of osmotically active substances at a time t,
V_0 = initial volume of osmotically active substances,
P_0 = initial osmotic pressure within the cell,
A = surface area of cell,
t = time taken for volume to change from V_0 to V_t.

Because there was no fixed relation between A and V it was not possible to use the further elaborations of this formula worked out by Lucké & McCutcheon (1932) to allow for changes in surface area during shrinkage. As the differences in values for permeability obtained by taking A first as the initial surface area and then as the final surface area amounted to about 10%, the error involved by taking A as the mean surface area during shrinkage was not very great.

The parasites were brought into equilibrium with a 0·7 M sucrose solution to determine V_0. P_0 was equivalent to the osmotic pressure of the 0·7 M sucrose solution. This solution was then replaced by a hypertonic solution of sucrose of known strength, and the volumes during and after shrinkage were recorded to obtain values for V_t and $V_{eq.}$; t was known and A was found as described. From

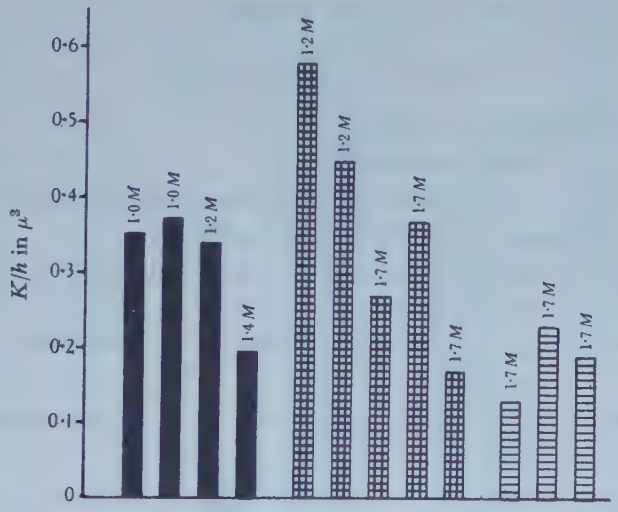

Fig. 3. Permeability to water: K/h. ■ K + Na present. ▦ Na present. ▤ Mg or Ca present.

a series of such experiments values for K/h were found. The concentration of the external medium was varied in one series of experiments to see whether this altered K/h, and in a second series of experiments the concentration of the hypertonic solution was kept constant but the cation present in the solutions was varied. The amount of the electrolyte present was negligible in its effect on the osmotic pressure of the sucrose solution, 0·1 ml. of 0·6 M NaCl or 0·4 M $CaCl_2$ or $MgCl_2$ being added to 20 ml. of sucrose solution.

The results are given in Fig. 3. Permeability is measured by the number of cubic micra passing through one square micron of the surface per atmosphere difference of pressure across the surface per minute. It varies from 0·06 to 0·58 of these units. There is a slight tendency for the permeability to be higher when the concentration of sucrose in the external medium is low and when Ca^{++} or Mg^{++} ions are present, but the number of experiments is not large enough to determine the significance of the difference. The results do show, however, that wide variations

in the conditions of the external medium make little difference to the permeability of the cell and, therefore, the values obtained are likely to be similar to the natural values.

THE PERMEABILITY TO NON-ELECTROLYTES

A method for estimating the permeability of a cell to non-electrolytes which penetrate slowly has been devised by Jacobs & Stewart (1932). When the cell is placed in an isotonic solution of a non-penetrating solute to which is added a known quantity of a non-electrolyte which penetrates the cell slowly, the volume is first diminished by the loss of water; then, as the non-electrolyte accumulates within the cell and raises the internal osmotic pressure, water re-enters the cell and its volume increases.

The formula for the rate of change of volume is

$$\frac{dV}{dt} = K_2 A \left[\frac{a+S}{V} - (C_m + C_s)\right], \qquad \text{(i)}$$

where K_2 = the permeability to water,

A = the surface area of the cell,

a = the amount of osmotically active substances in the cell expressed in units osmotically equivalent to C_m and C_s,

C_m = the concentration of the external medium before the addition of the penetrating substance,

C_s = the external concentration of the penetrating substance,

V = volume of the cell,

S = the amount of substance entering the cell in a time t expressed in the same units as a.

At the moment of minimum volume dV/dt is zero, in which case the above formula may be rewritten:

$$S = (C_m - C_s)\, V - a. \qquad \text{(ii)}$$

In (ii) V is the minimum volume. The formula for the rate of entry of the non-electrolyte is

$$K_1 = \frac{V}{At} \log_e \frac{CV}{CV - S}, \qquad \text{(iii)}$$

where t = time for S units of the non-electrolyte to enter the cell. Neither the volume nor the surface area are constant and, therefore, V and A in (iii) were taken as the mean volume and surface area during shrinkage. In one experiment with ethylene glycol the permeability was worked out, taking the volume and surface areas first as initial values and then as their minimum values. The permeability was then found to lie between 3·0 and 4·3 mol., and was therefore well within the range found in all the ethylene glycol experiments. In experiments on *Gregarina* the concentration of the penetrating non-electrolyte in the hypertonic solution was 1 mol. per litre, except in two of the mannitol experiments, in which 0·5 M concentrations of mannitol were used.

The details of the results are given in Fig. 4. The changes in volume in the various non-electrolytes are shown in Figs. 5 and 6. It will be seen that the initial decrease in volume and the time taken to establish equilibrium are inversely proportional to the rate of penetration of the solute.

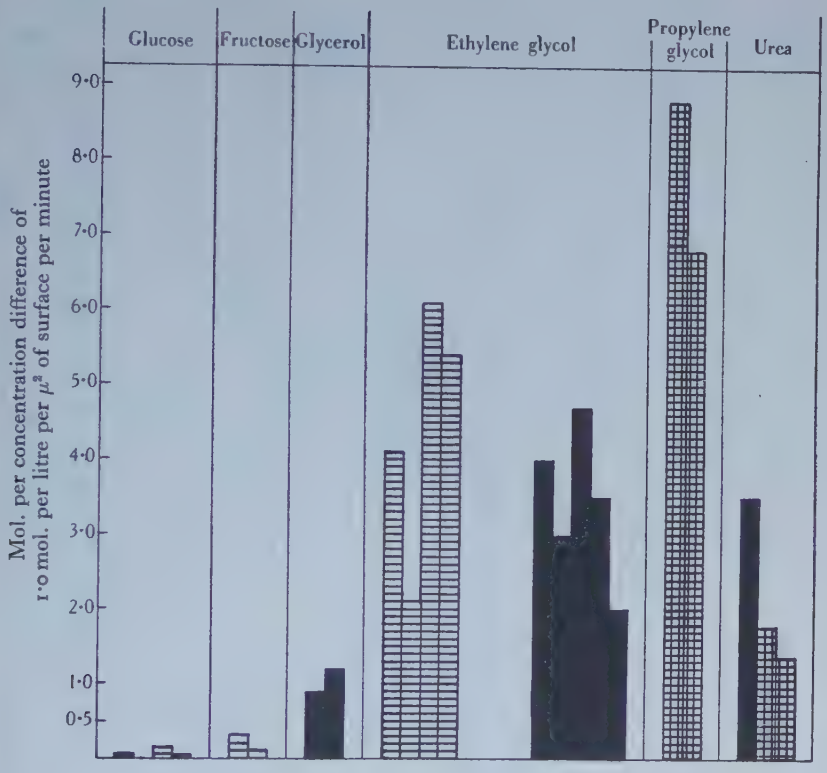

Fig. 4. Permeability to non-electrolytes. ■ Na + K present. ▦ Na present. ▤ Mg or Ca present.

PERMEABILITY TO ELECTROLYTES

The method used for these experiments was similar to that used for non-electrolytes. The parasites were irrigated for at least 30 min. in a 0·7 *M* sucrose solution and their volume in this solution recorded. The irrigating fluid was then changed for a solution of 0·4 *M* $CaCl_2$ dissolved in a 0·7 *M* sucrose solution or for a solution of 0·6 *M* NaCl dissolved in 0·7 *M* sucrose solution. The changes in volume in these osmotically equivalent solutions were recorded.

The changes in volume in these solutions are shown in Figs. 7 and 8. There is only a slight tendency for the volume to increase after the initial decrease, which suggests that *Gregarina* is relatively impermeable to sodium and calcium chloride. The experiments did not indicate whether the salts were penetrating as ions or molecules.

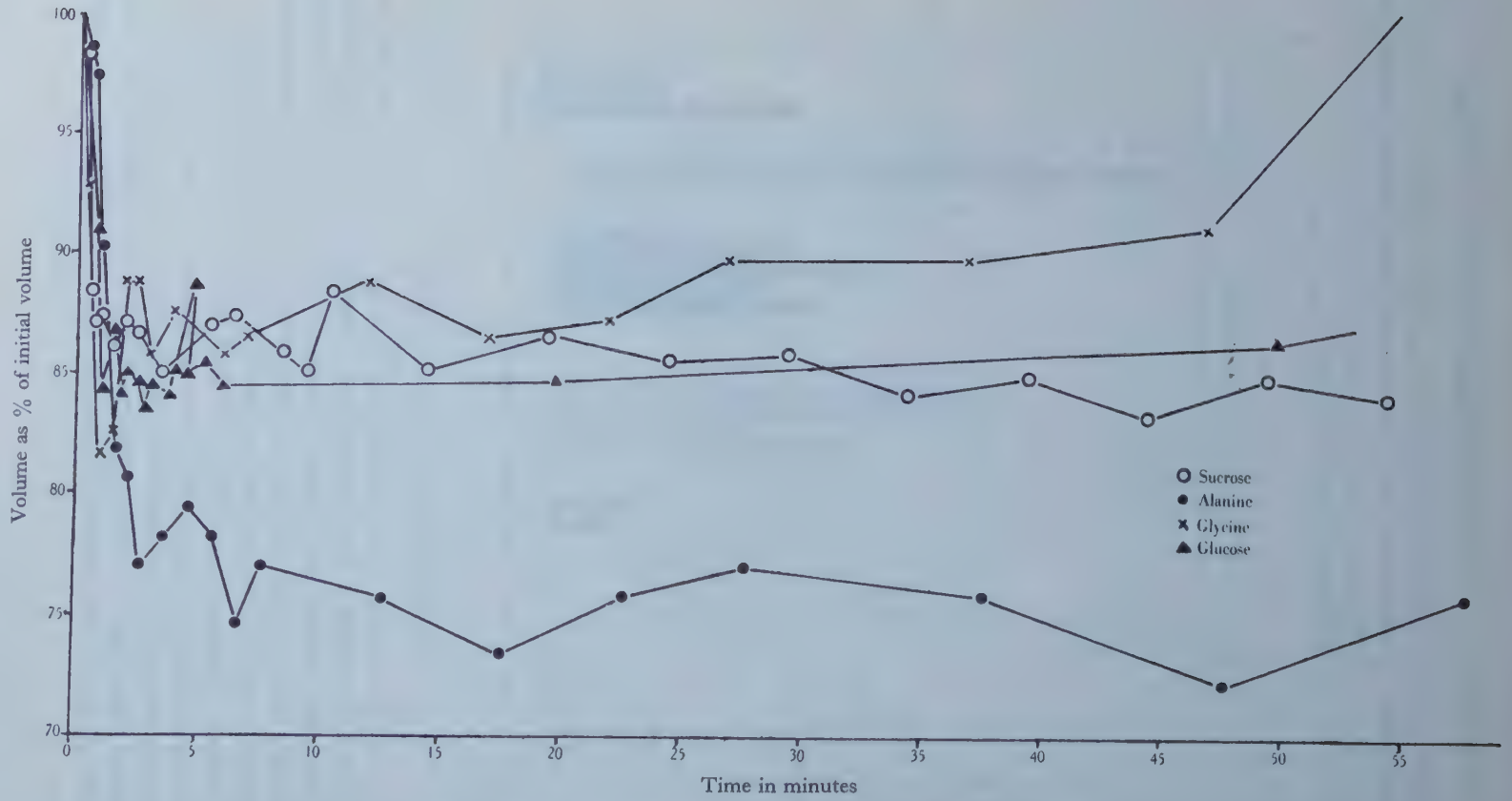

Fig. 5. Changes in volume in 1 *M* solutions of sugars and amino-acids.

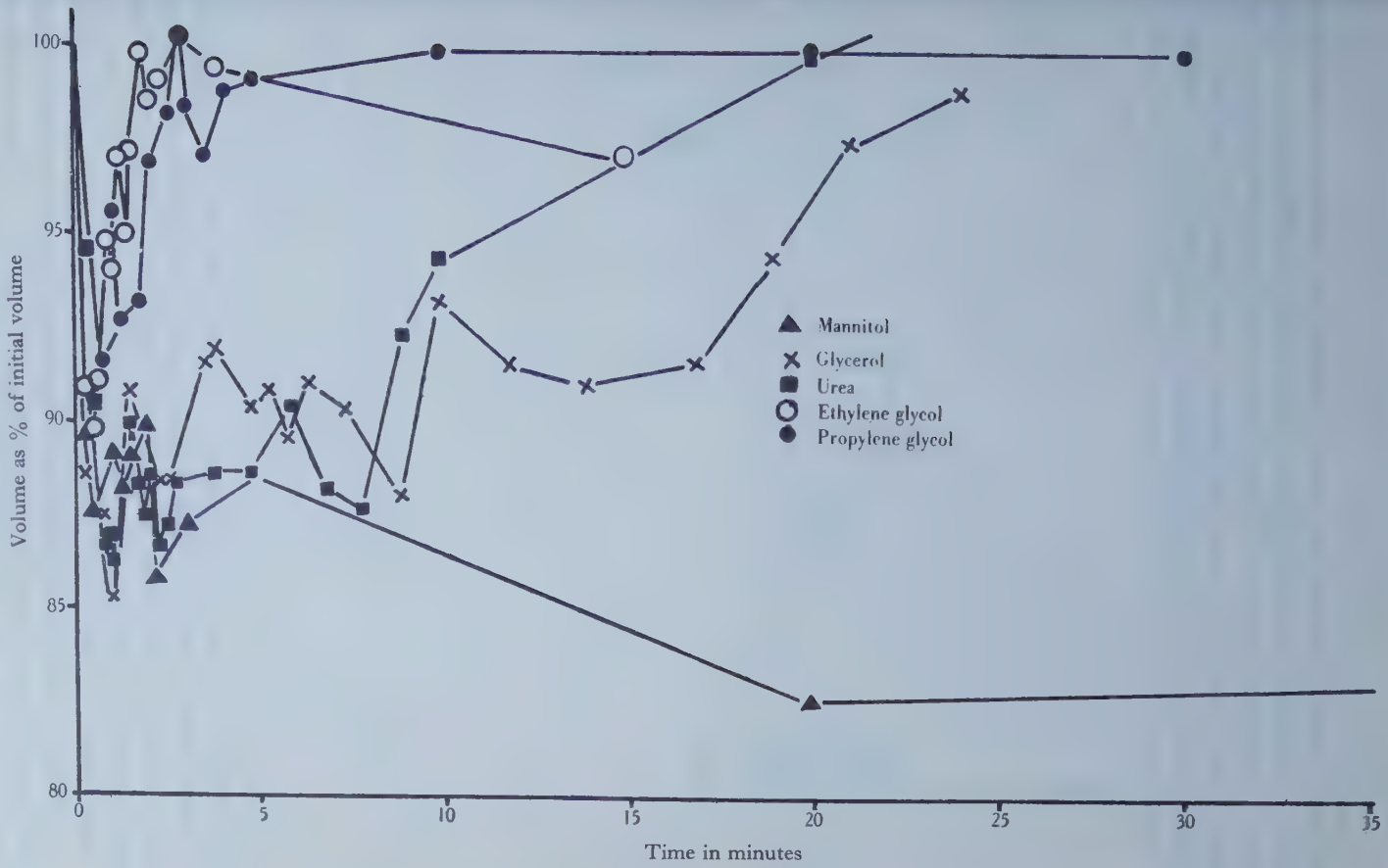

Fig. 6. Changes in volume in 1 *M* solutions of alcohols and urea, made up in 0·7 *M* sucrose solutions.

DISCUSSION

As already stated, *Gregarina* has no mouth and all food substances entering the cell must do so through the body surface. In this respect it differs from other cells studied. *Amoeba* and peritrich ciliates take in solid food, *Arbacia* eggs already contain the food required for early development and plant cells synthetize their food from simple inorganic compounds. The substances essential for life which the trophozoite must obtain are carbohydrates, proteins and fats. Carbohydrates are

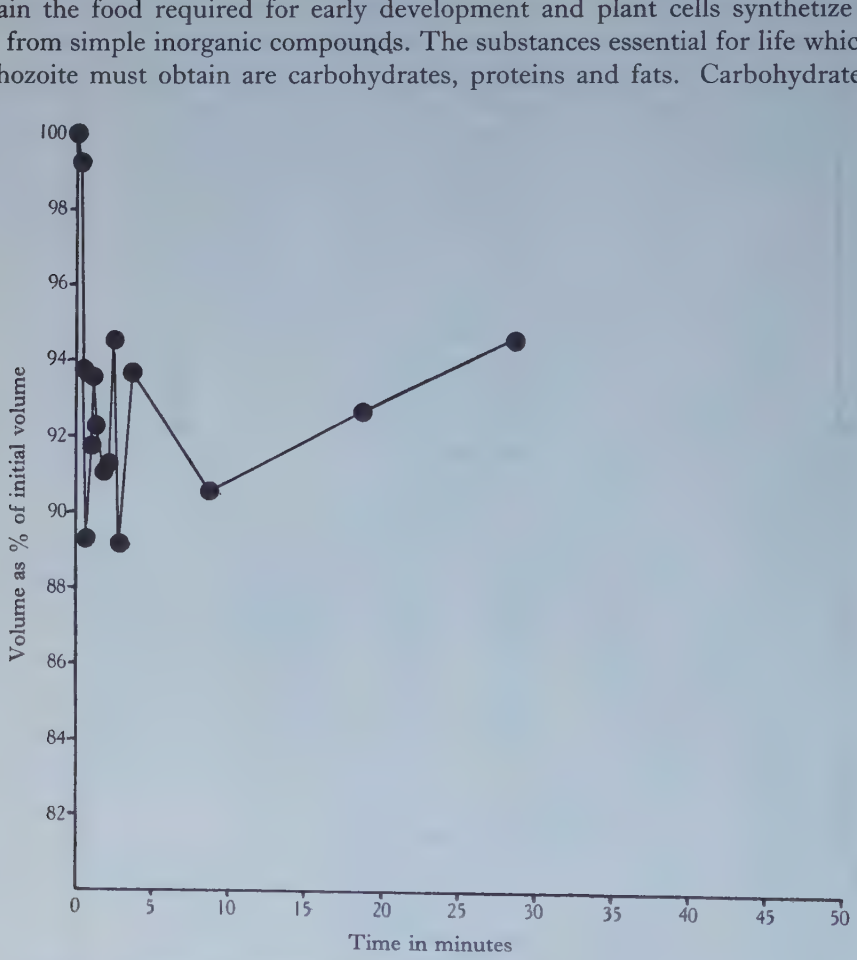

Fig. 7. Changes in volume in 0·4 *M* CaCl dissolved in 0·7 *M* sucrose solution.

probably available as monosaccharide sugars produced in the gut of the meal-worm by the action of its digestive enzymes on starch present in its food. Similarly, the material from which proteins and fats may be manufactured are probably available as amino-acids and as fatty acids and glycerol respectively.

Gregarina has no mechanism such as the contractile vacuole, present in many free-living Protozoa, for controlling its internal osmotic pressure. In all experiments the trophozoites quickly adjust their internal osmotic pressure to that of the external medium. This suggests that in their normal environment their internal osmotic

pressure fluctuates with that of the gut fluids of their host. Evidence in support of this was obtained when the meal-worms were fed on moist bread. The trophozoites were then much more swollen in appearance than when taken from meal-worms fed on dry bread. Normally the contents of the gut of the meal-worms are in a very viscous state. The osmotic pressure of the gut fluid appeared to be approximately equal to that of a 0·5 to 0·7 *M* sucrose solution since the gregarines showed a negligible change in volume on transference from the gut fluid to solutions of

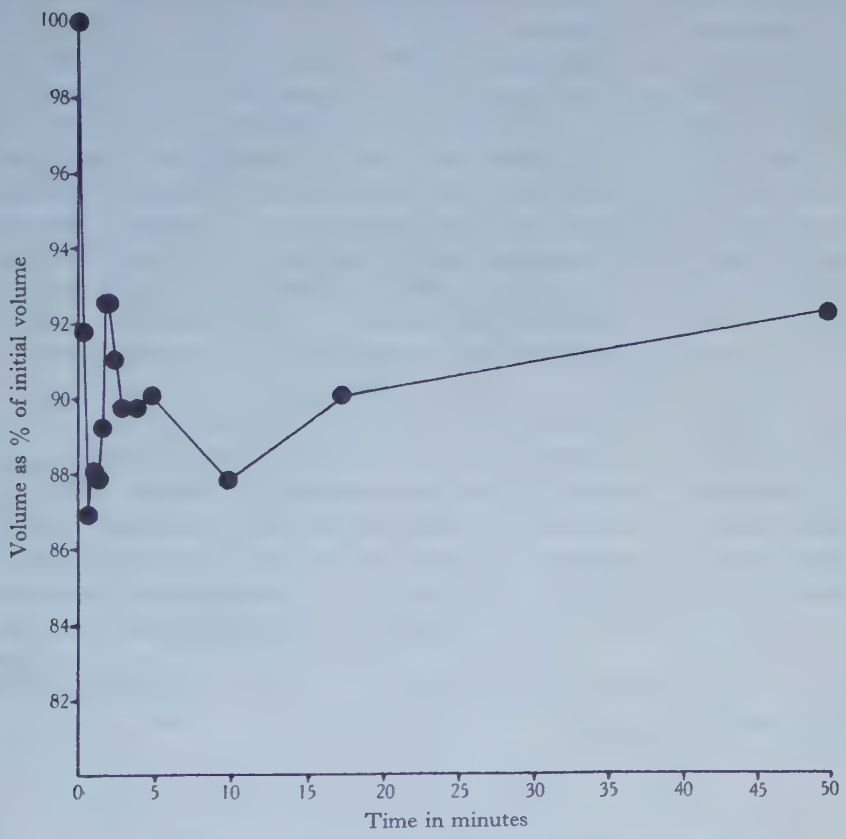

Fig. 8. Changes in volume in 0·6 *M* NaCl dissolved in 0·7 *M* sucrose solution.

these strengths. The osmotic pressure of the gut fluid was later measured on Prof. A. V. Hill's thermocouple (by the kind co-operation of the Department of Physiology of University College), and it was found to be approximately equal to that of a 0·8 *M* sucrose solution. The internal osmotic pressure of *Gregarina* is therefore normally much higher than that of a free-living fresh-water protozoan such as *Zoothamnion* which Kitching (1938) found to have an internal osmotic pressure equal to that of a 0·05 *M* sucrose solution.

Between 70 and 75 % of the volume of the trophozoite was found to be occupied by osmotically inactive substances, chiefly glycogen or paraglycogen. This estimate

agrees with the work of Daniels (1938). She centrifuged trophozoites *in situ* in the gut and then killed and fixed them rapidly and cut longitudinal sections. The granules of paraglycogen together with a small amount of fat and other solids became massed at one end and the densely packed granules occupied approximately two-thirds to three-fourths of the volume of the trophozoite. The value for other cells appears to be considerably lower. Lucké & McCutcheon (1932) found the osmotically inactive substances present in *Arabacia* eggs to occupy 7–14% of the total volume. From the results of Fauré-Fremiet (1924) they estimated the amount present in eggs of *Sabellaria alveolata* as 31% of the volume. Ege (1922) found the osmotically inactive substances in red blood corpuscles of rabbit to be 40% of the volume. However, a large amount of glycogen is characteristic of other parasites. In *Ascaris* the glycogen was estimated by Weinland (1900, 1901) to account for 25% of the dry weight, and other investigators (Rauther, 1925) have placed the amount as high as one-third of the body weight. The metabolism of *Ascaris* has not been satisfactorily worked out, but it has been suggested that the glycogen content is associated with the low oxygen pressure of the medium in which the worms live and which necessitates periods of anerobic respiration, although recent work (Davey, 1938) tends to minimize this possibility. It is of interest that *Gregarina* living under similar conditions should contain a high percentage of glycogen.

Comparison of permeabilities

Considering the great difference in environment between *Gregarina* and free-living Protozoa and *Arbacia* eggs and in the method of nutrition, there are surprisingly few differences in the permeability of the cell surface. Table 2 gives the known permeabilities to water and solutes for other cells and those obtained for *Gregarina*. It will be seen that the permeability to water is much the same for all except red blood corpuscles. *Gregarina* agrees with *Cothurnia* (Kitching, 1936) in

Table 2. *Showing the permeabilities of various cells to water and non-electrolytes*

The solutes are given in mol. $\times 10^{15}/\mu^2$/min./concentration difference of 1 mol. per litre. Water is given μ^3/μ^2/min./atm.

Cell	Water	Glucose	Glycerol	Ethylene glycerol	Urea	Propylene glycol	Reference
Red blood corpuscles of ox	2·5	—	0·011	0·96	108·0	—	Jacobs, 19
Arbacia eggs:							
(1) Unfertilized	0·14–0·23	—	—	3·3–5·6	—	6·7– 9·0	Stewart &
(2) Fertilized	0·31–0·59	—	—	5·4–9·2	—	11·5–14·3	Jacobs, 1
Arbacia eggs, unfertilized	—	No visible penetration	0·5	3·5	Very low	—	Stewart, 19
Free-living Protozoa:							
(1) *Amoeba proteus*	0·026	—	—	—	—	—	Mast & Fowler, 19
(2) *Cothurnia*	0·05–0·1	—	—	—	—	—	Kitching, 1
(3) *Zoothamnion*	0·125–0·25	—	—	—	—	—	Kitching, 1
Parasitic Protozoa, *Gregarina*	0·06–0·58	0·03–0·15	0·9–1·2	2·0–6·1	1·36–3·5	6·8–8·97	

being relatively impermeable to neutral salts. The permeability to lipoid soluble substances such as ethylene and propylene glycol is similar for *Arbacia* eggs (Stewart & Jacobs, 1936) and for *Gregarina*. All the cells are relatively impermeable to large inert molecules such as sucrose. An important difference is the permeability of *Gregarina* to glucose. Jacobs & Stewart (1932) report that dextrose shows no visible signs of penetration into *Arbacia* eggs over a period of several hours. In hypertonic solutions of glucose and fructose *Gregarina* shows a marked increase in volume in about 30 min. The permeability of *Gregarina* to glycerol is also higher than that of *Arbacia* eggs but the number of experiments performed was too few for the difference to be significant. Glycine entered at about the same rate as glucose, or possibly slightly faster. Alanine caused a reduction of the volume by a percentage greater than that produced by a sucrose solution. As the cell showed marked signs of a cytoplasmic contraction it was concluded that the effect was not entirely osmotic. Since the contraction was permanent it was impossible to determine whether alanine entered the cell. The only important modification shown by the cell surface of *Gregarina*, as compared with that of *Arbacia* eggs and free-living Protozoa, is therefore that it allows the passage of monosaccharide sugars.

Mechanism of permeability

Collander (1937) investigated the permeability of *Chara ceratophylla* to forty-five organic non-electrolytes and also found the partition coefficients of these substances between ethyl ether and water and between olive oil and water as a measure of their lipoid solubility. He came to the conclusion that there was a close correlation between permeability and lipoid solubility but that small molecules such as ethylene glycol penetrated the plasma membrane faster than would have been expected on account of solubility in oil alone. Stewart (1931) concluded that lipoid solubility was an important factor in the permeability of *Arbacia* eggs. In *Gregarina* substances of high lipoid solubility, such as propylene glycol, penetrate more quickly than substances of low lipoid solubility even although they may have a larger molecule than the latter. This suggests that lipoids are present at the cell surface and that solubility in fats is a decisive factor in affecting permeability.

Of substances of low lipoid solubility urea shows considerable variation in its power to penetrate cells. Red blood cells (Jacobs, 1934) show a very high permeability to urea and a low permeability to ethylene glycol, but *Gregarina*, *Arbacia* eggs (Stewart, 1931), and plant cells (Collander, 1937), are not so permeable to urea as they are to ethylene glycol. Particulars of the solubility of these substances in true lipoids are scarce. Some of the partition coefficients obtained by Collander & Bärlund (1933) are given below.

It will be seen that the position of urea in the series varies according to the mixture of liquids used. The lipoids present at the cell surface are probably very different from the solvents used to estimate lipoid solubility and these partition coefficients are therefore of little value for purposes of correlating permeability and lipoid solubility in the case of substances such as urea. It is possible that the variations found in permeability to urea are due to differences in the lipoids present

at the cell surface of different cells. Nirenstein (1920) found the power of dyes to stain *Paramoecium* was proportional to their solubility in a mixture of oil, oleic acid and diamylamine. Here the important factor is chemical affinity and not solubility, and this may be true for other substances also.

Substance	Partition coefficients	
	Olive oil/water	Olive oil and oleic acid/water
Mannitol	—	<0·00001
Glucose	—	<0·00001
Fructose	—	<0·00001
Glycerol	0·00007	0·00015
Urea	0·00015	0·0052
Ethylene glycol	0·00049	0·0016
Propylene glycol	0·0057	0·0098

Molecular size may be an important factor controlling the entry of substances of low lipoid solubility. The molecule of mannitol is only slightly larger than that of glucose or fructose, but in hypertonic solutions of glucose a marked increase in volume occurred in 40–50 min., whilst in hypertonic solutions of mannitol no increase in volume occurred after 90 min. The discrimination between these substances may be merely due to differences in the size of molecule or it may be due to active uptake of glucose such as occurs in the lumen of the gut of mammals (Auchinachie *et al.* 1930; Donhoffer, 1935; Verzár, 1935). Schmengler & Höber (1933) found that the kidney tubules of the frog were impermeable to mannitol. In its action as a molecular sieve the cell surface may be compared with artificial membranes such as copper ferrocyanide membrane used by Collander (1924). It has been suggested that pores are present in the cell surface through which substances of low lipoid solubility can pass, and that the diameter of the pores determines which molecules shall pass through. If substances of low lipoid solubility do enter by such means, then the pores must be larger in *Gregarina* than in *Arbacia* eggs to allow the passage of glucose in the former. In work on permeability there has been a tendency to ignore the shape of the molecule. The rate at which molecules, which have a long straight chain of atoms, can pass through pores is probably very different from the rate at which molecules of the same weight, but which have the atoms arranged in a more compact form such as a ring, can pass through. Harvey & Danielli (1938) cited a third factor of importance. They state that to pass through a lipoid membrane, or pore structure of mesh size approximately that of the penetrating molecule, the latter must have kinetic energy greater than that of the potential barrier. This factor would probably be of importance in governing the entry of glucose.

The structure of the surface of *Gregarina* appears to be similar to that of other cells which have been studied except in that part of it which is responsible for allowing the passage of large molecules of low lipoid solubility.

SUMMARY

Gregarina contains a large amount of osmotically inactive material, chiefly glycogen. In this respect it resembles other parasites such as *Ascaris*. It possesses no mechanism for regulating its internal osmotic pressure; therefore it quickly reaches a state of equilibrium with the medium in which it is placed. It is more permeable to substances of high lipoid solubility than to those of low lipoid solubility even though the former may have a larger molecule than the latter. In this respect it resembles other cells which have been studied, but it is more permeable than these cells to substances of low lipoid solubility and large molecular size such as glucose and fructose.

I wish to thank Prof. H. G. Jackson in whose Department at Birkbeck College, University of London, this work was carried out. I am indebted to Dr J. A. Kitching for suggesting the problem and for helpful criticism during the course of the work. Also to Mr Alastair Graham for continuous interest and advice.

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